JOURNAL OF THE NATIONAL CANCER INSTITUTE

National Institutes of Health • Public Health Service

Volume 25 • Number 3

September 1960

Some Peculiar Biological and Biochemical Properties of a Mouse Hepatoma Induced by Chrysoidin. I. Biological and Biochemical Characteristics of the Hepatoma [1, 2]

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SUMMARY

A transplanted hepatoma of the mouse is described. The neoplasm arose in the liver of a C57BL mouse after prolonged feeding of chrysoidin (2,4′-azodianiline). It was transplantable into only strain C57BL mice and their F_1 hybrids from crosses with strains RIII and A. Thirty-four passages have been performed with 80 to 100 percent takes. About 55 percent of the mice had metastases, almost exclusively to the lungs. Histologically, the neoplasm resembled those described by other authors except that intranuclear inclusions were more conspicuous and more frequent. In some instances Kupffer cells, the cytoplasm, and intranuclear inclusions of hepatoma cells contained brown protoporphyrin pigment.—J. Nat. Cancer Inst. 25: 443–453, 1960.

IN AN earlier paper (*1*) we reported that when hepatoma was induced in strain C57BL mice fed chrysoidin (2,4′-azodianiline), 24 percent developed adenomas and 48 percent developed hepatomas. The results of transplantation of this hepatoma and some of the morphological and biological characteristics of the transplants are presented. The two papers which follow will deal with some of its metabolic characteristics.

EXPERIMENTS

The primary hepatoma induced by chrysoidin was transplantable into strain C57BL mice and their F_1 hybrids from crosses with strains RIII or A. In the first and second passages the percentage of takes was 30; in subsequent passages it was 80 to 100.

The latent period of the neoplasm averaged 147 days on the first passage but only 49 days in subsequent passages. Death of the animal due to neoplastic growth occurred at an average of 202 days after the

[1] Received for publication January 19, 1960.
[2] Presented at the Seventh International Cancer Congress, London, England, July 7, 1958.
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first passage, and in subsequent passages, within 95 days after implantation. At the time of death the size of the growth was about 4630 mm.^{3}

As of October, 1959, this tumor was in the 34th transplant generation, and had been transplanted into 457 mice of both sexes 2 to 3 months of age; it took in 343 mice, or 75 percent.

The malignancy of the hepatoma was evidenced by the presence of metastases in the internal organs, especially in the lungs where they were found in about 55 percent of the mice, whereas in the spleen, liver, and lymph nodes only an occasional metastasis was observed. Metastases were macroscopically visible in about 23 percent of the mice and in the rest, only by microscopic examination.

No metastases were observed before the 56th day after transplantation; they appeared mostly between the 63d and 126th days—on an average, by the 84th day after transplantation of the neoplasm. A large percentage (30%) of the animals killed between the 63d and 126th day after transplantation had no detectable metastases.

The transplanted hepatoma did not differ markedly in its microscopic structure from the primary chrysoidin-induced hepatoma described in a previous paper (*1*). It should be emphasized that contrary to the experience of Andervont and Dunn (*2*, *3*) the morphological pattern of our hepatoma remained unchanged during transplantation.

Three types of morphological structures were found: one pattern resembling hepatic trabeculae (hepatoma trabeculare), another consisting of polymorphous cells, and a third characterized by the presence of dilated sinusoids. Almost invariably all three occurred in the same tumor. The cells of the hepatoma trabeculare resembled normal hepatic trabeculae but did not show a lobular arrangement. The tumor trabeculae formed parallel bands and occasionally contained accumulations of polymorphous cells, the phenomenon termed by Andervont and Dunn as "a tumor within a tumor" (*2*). We never have found cholangiocarcinomatous patterns.

The cytoplasm of the hepatoma cells usually contained basophilic clumps, like that of liver cells, and had a slightly foamy appearance; sometimes it was coarsely vacuolated. The vacuoles sometimes stained with Best's method, indicating the presence of glycogen; mostly, however, they were unstained. The nature of the vacuolization has not yet been elucidated. Staining with Sudan III was usually negative. Glycogen and fat were more frequently detected in areas devoid of vacuolated cytoplasm.

We have already mentioned the accumulation of polymorphous cells in the trabecular structures of the hepatoma. Elsewhere in the tumor, extensive areas consisting exclusively of polymorphous cells were observed, the extent and degree of their polymorphism varying from place to place (fig. 1). The polymorphous cells reached a considerable size, many times larger than the normal liver cells. Their cytoplasm was basophilic and exhibited round, clear zones or unstained vacuoles. The cytoplasm contained several vacuoles that tended to coalesce. Basophilic granules

were observed in some of the vacuoles. In other instances the cytoplasm of the hepatoma cells was filled with pale rose-staining hyaline globules. The cytoplasm of the polymorphous cells frequently showed rings of finely basophilic granules and, occasionally, bundles of strongly basophilic filaments, which were arranged in parallel bundles or crossed one another obliquely (fig. 2). Szanto and Popper (*4*) and Andervont and Dunn (*2*) believed that the basophilia may be due to ribonucleic acid. The polymorphous cells contained one and, not infrequently, several nuclei which were many times larger than those of the hepatoma trabeculare and usually hypochromatic.

One to three or more intranuclear inclusions were usually present in the tumor cells (figs. 3 to 6). In almost every transplanted hepatoma, especially in polymorphous cells, we observed one or several intranuclear inclusions. These often reached a giant size and were vacuolated (figs. 3 to 5). We also found necrotic lesions that were scarcely visible macroscopically and occasionally contained calcium deposits. Frequently the necrotic zones were fibrotic. No glandular tubules that might suggest cholangiocarcinoma were found; occasionally structures resembling tubules were seen, but they were clearly the result of degeneration of neoplastic cells.

Occasionally a lobular arrangement of hepatic cells was observed which was due to the separation of cell groups by sinusoids (fig. 7). Central veins or portal tracts were not found in these "lobules." Although the sinusoids in figure 7 are devoid of lumens, each tumor showed portions with more dilated sinusoids (fig. 8), the lumens of which were filled with either homogeneous protein fluid or erythrocytes.

The lung metastases (fig. 9) did not differ histologically from the primary tumor, except that dilated sinusoids were rarely seen; vacuolization of cell cytoplasm was frequent. Spleen and liver metastases were rare and showed a different morphological pattern. Vacuolated intranuclear inclusions were also observed in lung metastases.

In paraffin sections of a few hepatomas, brownish-yellow pigment was present in the cytoplasm and, rarely, in the nuclei and intranuclear inclusions of the tumor cells. This pigment was seen mainly in large polymorphous cells in which the whole cytoplasm was filled with pigment granules, so that the nucleus was pushed to the periphery and was scarcely visible. The pigment appeared either as fine dust, fine granules, or globules of various shapes and sizes. The intensity of the brown hue depended on the condensation of the granules or the size of the globules. Frequently, the pigment granules were situated in small or large vacuoles. Stein's test (*5*) for bilirubin was negative. Staining by Tirmann-Schmelzer's method (*6*) failed to show any iron in the granules.

DISCUSSION

The intranuclear inclusions of the chrysoidin-induced hepatoma appear to be larger and more vacuolated than those described in other hepatomas.

Some of the large structures resemble nucleoli but may be intranuclear inclusions similar to those observed in glioma (*7*), meningioma, perineural fibroblastoma, hemangioblastoma (*8*), cultures of meningioma (*9*), and the nuclei of non-neoplastic cells, such as fetal leptomeninges (*10*) and mouse (*11*) and human liver cells. We have also found changes suggestive of intranuclear inclusions in livers of mice fed chrysoidin for 2 weeks and in livers of human beings with tumors (*12*). These changes were, however, far less intense than those encountered in the hepatoma.

According to the literature, the brown and brownish-yellow pigment observed by us in hepatomas was rarely seen by other investigators. Lewis reported "bilirubin crystals" in monocytoma (*13*) and Dunn observed "hematoidin crystals" in reticulum-cell sarcoma (*14*). In contrast to our observations, no one has found pigment in nuclei. Lewis has not proved that the crystals observed by him are really bilirubin, though he refers to them as such. Dunn has tried to identify the crystals she observed and emphasized that they were, like hematoidin, rhomboid and therefore different from those we observed. The results of several chemical tests [Gmelin's, Ehrlich's diazo reaction, trichloroacetic acid, and Rosin's iodine (*14*)] and spectroscopic examinations did not suggest their identity with bilirubin. Dunn suggested that the commercial bilirubin with which the round crystals were compared might have been contaminated—a probability which she regarded as a plausible explanation for the divergence of results. She was, however, unable to explain why some very sensitive bilirubin tests were negative. In spite of this, Dunn assumed that the crystals observed were hematoidin, which is thought to be identical with bilirubin.

Further studies by Mędraś (*15*) indicate that the pigment observed in the chrysoidin-induced hepatoma is a protoporphyrin.

The hepatomas reported here differed from other hepatomas in having a low rate of aerobic glycolysis similar to normal liver (*16*). Another distinguishing feature of this hepatoma was the presence of glucose-6-phosphatase (*17*). The reasons for these differences and their correlation with intranuclear inclusions resulting in enlargement and vacuolization and in the presence of porphyrins remain to be determined. It is likely that further studies now in progress will contribute much to elucidation of the problem.

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PLATES

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Figures 1 through 9 stained with hematoxylin and eosin.

PLATE 55

FIGURE 1.—Transplanted mouse hepatoma. *Top:* hepatoma trabeculare, *bottom:* polymorphous cells with vacuolated cytoplasm. × 130

FIGURE 2.—Cytoplasm of the hepatoma cells with bundles of basophilic filaments. × 760

FIGURE 3.—Giant polymorphous hepatoma cells with large nuclei containing several intranuclear inclusions. *Bottom:* cells of hepatoma trabeculare. × 520

FIGURE 4.—Polymorphous hepatoma cells with vacuolated cytoplasm. Large nucleus containing vacuolated nuclear inclusions. Nuclear chromatin is sparse. × 760

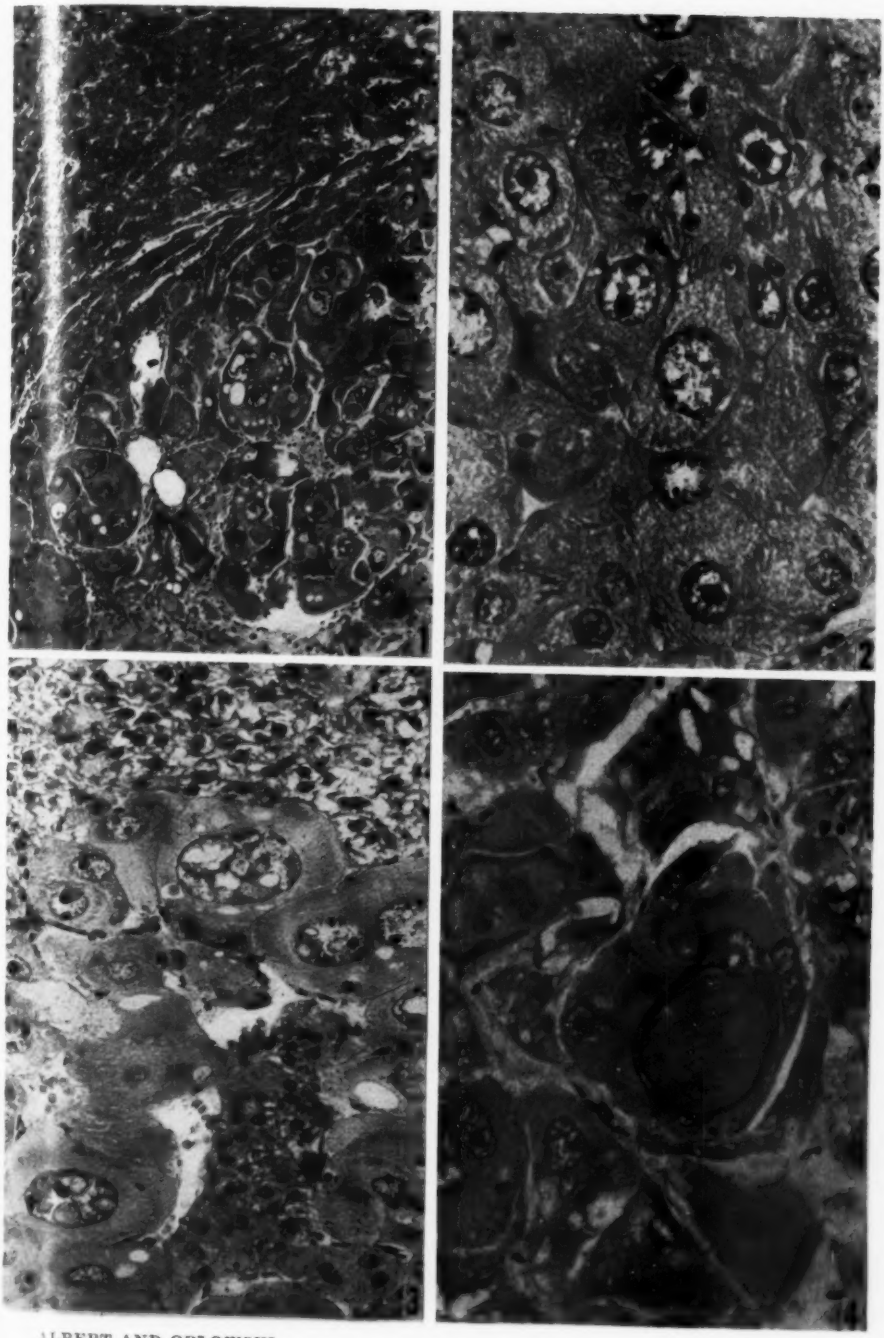

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PLATE 55

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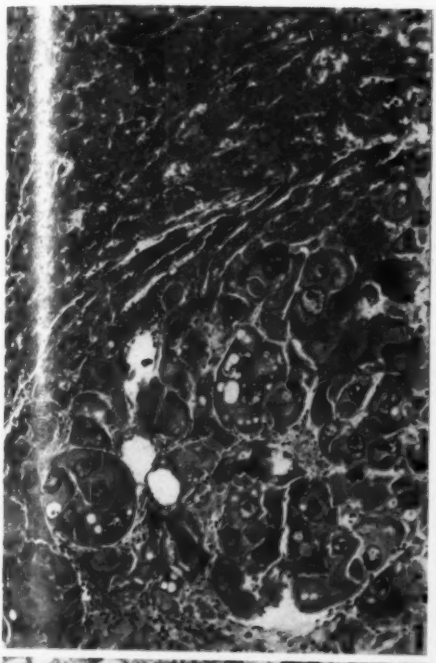

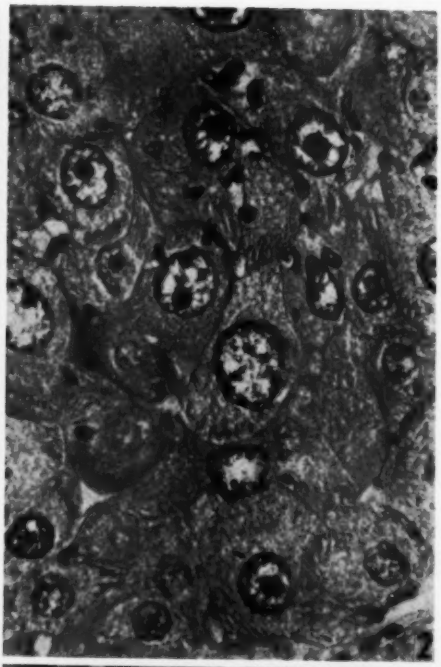

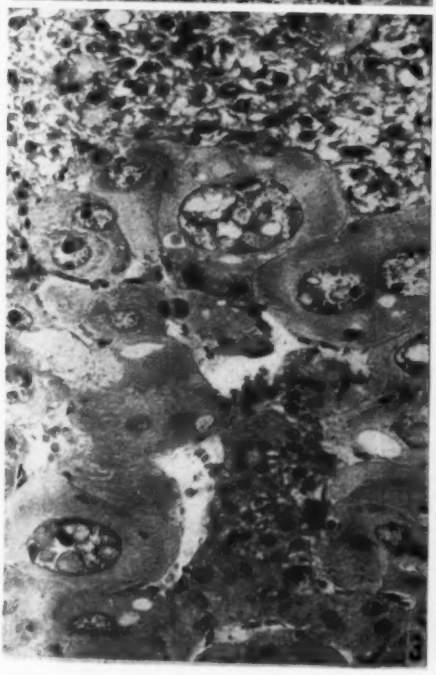

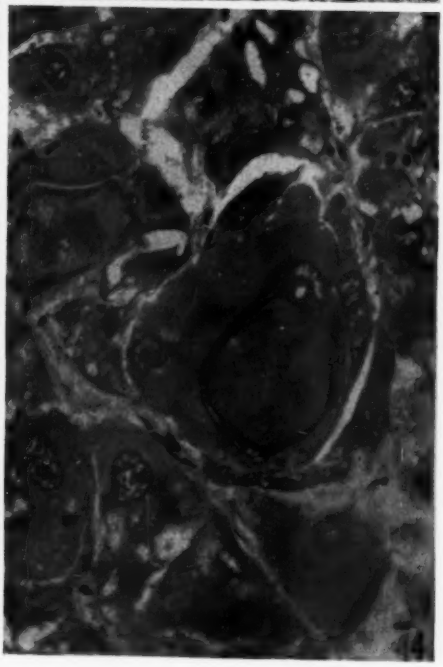

PLATE 56

FIGURE 5.—Highly vacuolated cytoplasm of a giant cell. Also vacuolated nucleus with condensed chromatin which contains a vacuole with nuclear inclusions. × 760

FIGURE 6.—One of the polymorphous giant hepatoma cells showing ringlike nuclei distorted by nuclear inclusions. × 520

FIGURE 7.—Transplanted mouse hepatoma. Distinct lobular pattern suggesting that of the liver. × 130

FIGURE 8.—Dilated sinusoids, some of them containing erythrocytes. × 130

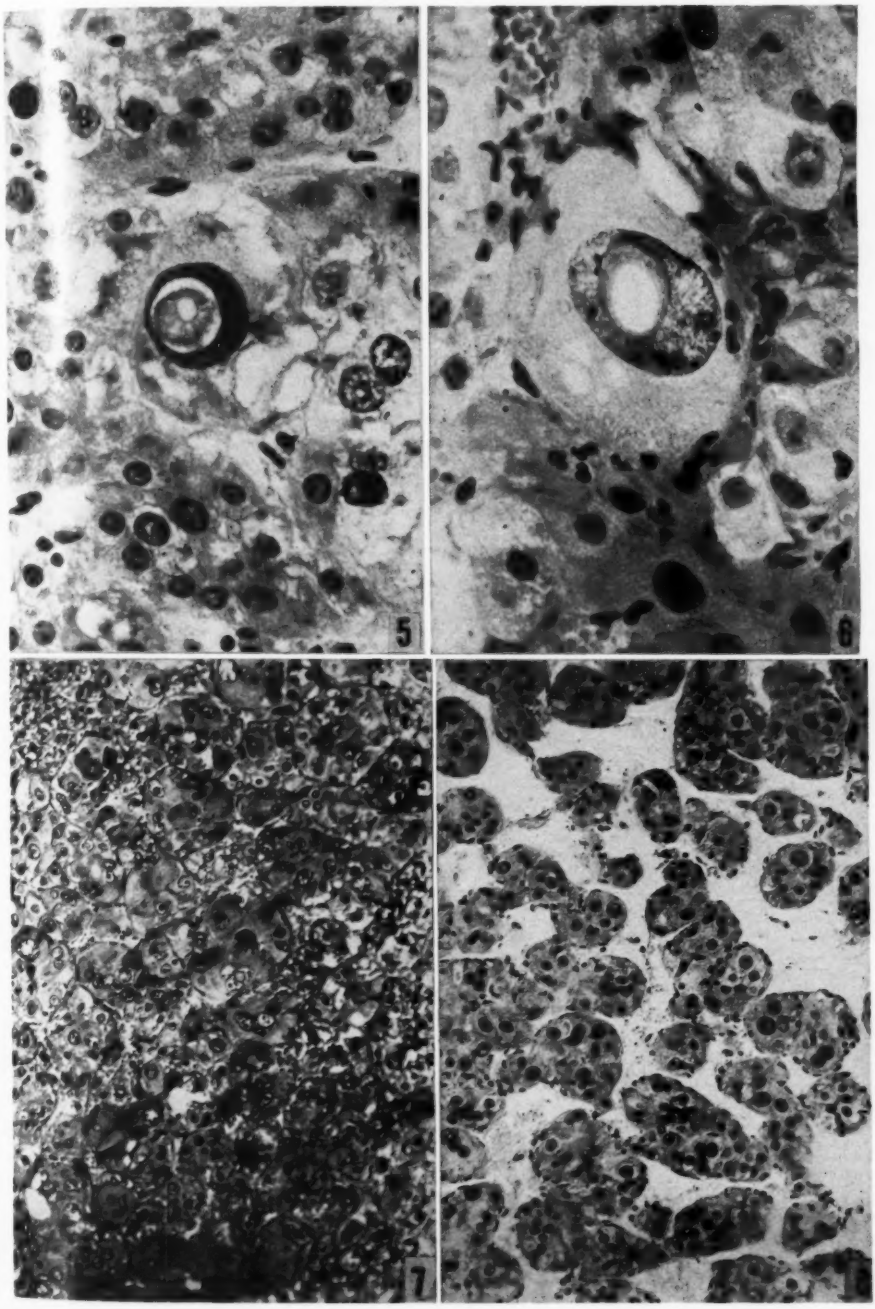
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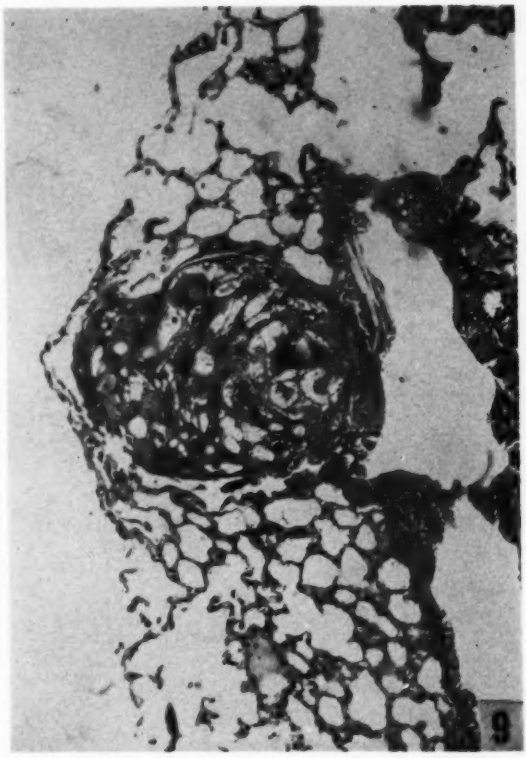
PLATE 57

FIGURE 9.—Transplanted hepatoma. Lung metastasis. Pronounced vacuolization of the neoplastic cells. × 130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Some Peculiar Biological and Biochemical Properties of a Mouse Hepatoma Induced by Chrysoidin. II. Metabolic Properties of the Hepatoma [1, 2]

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SUMMARY

The metabolism of slices of a transplanted hepatoma originally induced with chrysoidin (2,4′-azodianiline) in C57BL mice was compared with that of liver slices from normal animals of the same strain. The metabolism of the hepatoma is characterized by a high respiratory rate, a high anaerobic glycolytic rate, and a low aerobic glycolytic rate no different from that of normal liver. This metabolic behavior is indicative of a low Warburg ratio. High aerobic and anaerobic glycolytic rates were observed in other tumors examined, *i.e.*, Crocker sarcoma 180, a spontaneous mammary adenocarcinoma of mice, a primary rat hepatoma, and a transplanted hamster hepatoma.—J. Nat. Cancer Inst. 25: 455–460, 1960.

SINCE WARBURG'S studies on respiration and glycolysis in slices of neoplastic tissue (*1*), there have been many attempts to detect some metabolic characteristics of malignant tissue that differ from normal ones. Though no metabolic reactions inherent only in neoplasms have been established so far, several features can be cited that are characteristic of most malignancies. It has been ascertained that malignant tumors are characterized by extensive aerobic and anaerobic glycolysis as well as by normal or occasionally reduced oxygen consumption. In neoplastic tissue glycolysis does not cease even in the presence of oxygen; hence the frequency of an incomplete Pasteur effect and a high Warburg ratio. The Meyerhof quotient is normal, about 1 to 2, which, as in muscle tissue, means that 1 molecule of oxygen absorbed by the neoplastic tissue causes the disappearance of 1 to 2 molecules of lactic acid.

Many investigators confirmed Warburg's results in their studies on the metabolism of other tumors (*2–5*). Cori and Cori demonstrated, moreover, that high glycolytic rates are found in tumor slices not only *in vitro* but *in vivo* as well (*6*). Some contrary observations were made by Murphy

[1] Received for publication January 19, 1960.
[2] Presented at the Seventh International Cancer Congress, London, England, July 7, 1958.
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and Hawkins (*7*), who found that in some spontaneous tumors of mice there is little aerobic glycolysis.

Berenblum *et al.* (*8*) investigated the oxygen uptake, aerobic and anaerobic glycolysis, and respiratory quotient (R.Q.) of normal-skin epithelium and Shope papilloma and obtained almost identical results for both tissues. They concluded that aerobic glycolysis and a low R.Q. do not represent a disturbance characteristic of tumor growth.

In this paper we present evidence that transplants of the chrysoidin-induced hepatoma described by Albert (*9*), in C57BL mice, is characterized by an aerobic glycolysis which is not different from that found in liver slices from the same strain. Some biological properties of this tumor are presented in our other papers (*10*, *11*).

MATERIALS AND METHODS

The experiments were carried out on a transplanted hepatoma originally induced by chrysoidin (2,4′-azodianiline) in strain C57BL mice, on livers of normal mice of the same strain, and on the following neoplasms: Crocker sarcoma 180, a spontaneous mouse mammary adenocarcinoma, a primary rat liver carcinoma induced by 4-dimethylaminoazobenzene,[5] and a transplanted hepatoma of hamsters. (The hamsters were obtained through the courtesy of Dr. Alexander Haddow, Director, The Chester Beatty Research Institute, London, England.)

Routine manometric Warburg technique on 0.2 to 0.3 mm. thick tissue slices was used for metabolic measurements. Only those slices free of necrosis were used. Oxygen consumption was measured in Ringer's solution buffered with 0.067 M phosphate (*p*H 7.4) in vessels in which the carbon dioxide produced was absorbed by NaOH. To enlarge the absorption surface, scraps of filter paper were placed in the center well with the NaOH. Aerobic glycolysis was determined in Ringer's bicarbonate solution saturated with 5 percent CO_2 and 95 percent O_2, to which glucose was added to a final concentration of 0.2 percent. Lactic acid production was calculated from manometer readings corrected for both oxygen consumption and evolution of respiratory CO_2. Anaerobic glycolysis was measured in Ringer's bicarbonate solution saturated with 95 percent N_2 and 5 percent CO_2. Calculation of the results was based on the dry weight of tissue.

Aerobic glycolysis was also measured in serum saturated with 5 percent CO_2 and 95 percent O_2, and manifested itself by the increase in pressure of CO_2 released from serum bicarbonates by the lactic acid produced. If aerobic glycolysis did not occur, the tissue respiration caused a reduction of pressure in the manometer.

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[5] *Chemical Abstracts'* nomenclature: *N,N*-dimethyl-*p*-phenylazoaniline.

RESULTS AND DISCUSSION

Mean values for oxygen consumption (Q_{O_2}), aerobic glycolysis ($Q_A^{O_2}$), and anaerobic glycolysis ($Q_A^{N_2}$) are given in table 1. From these figures the Warburg ratio, the percentage of Pasteur effect, and the Meyerhof quotient were calculated.

The high anaerobic glycolytic rate of this hepatoma, the very low aerobic glycolytic rate, and the comparatively high oxygen consumption give rise to characteristic behavior of the Warburg ratio and the Pasteur effect. The Warburg ratio given in table 1 is 0.09, which does not differ appreciably from the value obtained for liver slices. It seems that the respiration of this hepatoma is sufficient to suppress glycolysis entirely. This observation was confirmed by experiments in which the inhibition of respiration by 0.001 M hydrogen cyanide yielded a glycolytic rate equal to that measured under anaerobic conditions. The figure for the Pasteur effect is very high for this tumor—96 percent. The figure for the Meyerhof quotient is the same as that noted for other tumors.

For the other tumors studied (*see* Materials and Methods), the metabolic characteristics were in accord with those observed in tumor tissue by Warburg, *i.e.*, high aerobic and anaerobic glycolytic rates and a high Warburg ratio.

The lack of aerobic glycolysis in our hepatoma was confirmed by experiments in which serum saturated with 5 percent CO_2 and 95 percent O_2 was used as the glycolytic medium. As text-figure 1 shows, in the absence of aerobic glycolysis, the respiration of these slices caused a reduction of pressure in the manometric vessels, a result previously noted in work with mouse liver and kidney slices. In parallel experiments with slices of Crocker sarcoma 180 and hamster hepatoma transplants, marked aerobic glycolysis brought about an increase of pressure in the manometric system.

Oxygen consumption is markedly higher in hepatoma slices than the slices of normal liver. No increase of aerobic glycolysis was noted in the hepatoma slices as compared with that in liver. In many instances the aerobic glycolysis of the hepatoma tissue was essentially equal to zero. The anaerobic glycolytic rate in the hepatoma is high, on the average ten times higher than that in normal liver slices. In this respect, this tumor behaves very much like other malignancies.

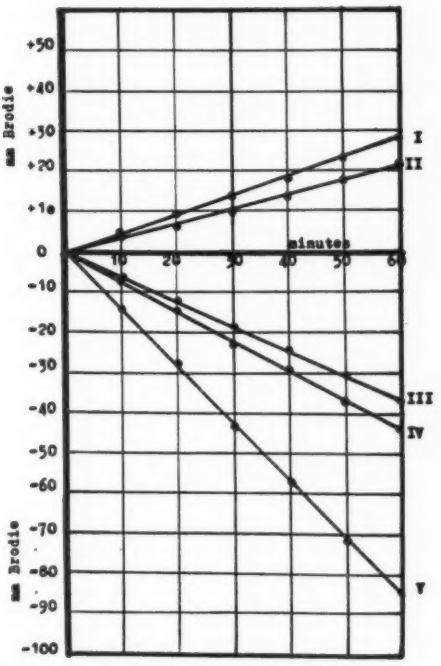
Our results indicate that the metabolism of the chrysoidin-induced hepatoma is distinct from that of other malignant tumors. It is characterized by a high respiratory rate, slight or absent aerobic glycolysis, similar to that observed in slices of normal liver, a high anaerobic glycolytic rate, and a high Pasteur effect. It is of interest that throughout a 3-year period, a low aerobic glycolytic rate has been observed consistently, and even after repeated passage this characteristic remains unchanged. Because its glycolysis is greatly suppressed in the presence of oxygen, this tumor is particularly suitable for investigation of the mechanism of the Pasteur effect.

These metabolic characteristics of C57BL mouse hepatoma might account for its relatively slow growth. They may be related to its con-

TABLE 1.—Metabolic measurements on normal liver and on chrysoidin-induced hepatoma from strain C57BL mice

Tissue	Oxygen consumption* Q_{O_2}	Aerobic glycolysis* $Q_A^{O_2}$	Anaerobic glycolysis* $Q_A^{N_2}$	Warburg ratio $\frac{Q_A^{O_2}}{Q_{O_2}}$	Pasteur effect (*percent*) $\frac{Q_A^{N_2}-Q_A^{O_2}}{Q_A^{N_2}} \times 100$	Meyerhof quotient $\frac{Q_A^{N_2}-Q_A^{O_2}}{Q_{O_2}}$	Number determinations
Normal liver	9. 5±0. 32	0. 91±0. 03	2. 85±0. 19	0. 1	68	0. 2	28
Chrysoidin-induced hepatoma	11. 6±0. 28	1. 02±0. 16	26. 6±0. 72	0. 09	96	2. 2	18

*Figures represent mean ± standard error.



TEXT-FIGURE 1.—Behavior of different tissue slices in serum saturated with 95 percent O_2 and 5 percent CO_2. Warburg vessels: V = 17.0 cm.^{3}; V_s = 13.0 cm.^{3}; I = Sarcoma 180; II = hamster hepatoma transplants; III = chrysoidin-induced hepatoma transplants; IV = liver of normal mice; V = kidney of normal mice. All values are calculated for 10 mg. of tissue, dry weight.

siderable differentiation in histological structure, which resembles that of the liver, though, in all other respects, it has the features of a neoplastic growth, *e.g.*, it metastasizes to the lungs in more than 50 percent of occurrences.

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Some Peculiar Biological and Biochemical Properties of a Mouse Hepatoma Induced by Chrysoidin. III. Activity of Glucose-6-phosphatase [1, 2]

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SUMMARY

The activity of glucose-6-phosphatase was investigated in homogenates of liver and of transplants of a chrysoidin-induced (2,4′-azodianiline) hepatoma in C57BL mice, as well as in other malignant tumors. Normal glucose-6-phosphatase activity was found in homogenates of chrysoidin-induced hepatoma as compared with that found in livers of C57BL mice. Lack of glucose-6-phosphatase activity, or only negligible dephosphorylation of glucose-6-phosphate, was observed in Crocker sarcoma 180, a spontaneous mouse mammary adenocarcinoma, primary rat liver carcinoma, and in transplants of hamster hepatomas.—J. Nat. Cancer Inst. 25: 461–464, 1960.

IN THE two preceding papers (*1*, *2*) we reported some biological and metabolic properties of transplants of a hepatoma induced by Albert (*3*), using chrysoidin (2,4′-azodianiline) in C57BL mice. It was found that the metabolism of this hepatoma differs from the generally accepted patterns for malignant tumors, especially with respect to aerobic glycolysis.

Since the formation and metabolism of glucose-6-phosphate are probably essential for the regulation of glucose metabolism, we have investigated the enzymes acting on this compound, using transplants of chrysoidin-induced mouse hepatomas as the biologic system. In these studies, we observed a second feature of this tumor that distinguishes it from other neoplasms, namely, the presence of glucose-6-phosphatase, an enzyme specific for the hydrolysis of glucose-6-phosphate (*4–8*). In splitting this ester into glucose and inorganic phosphate this enzyme is conceivably of great importance in the maintenance of the blood-glucose level. Glucose-6-phosphatase has been found in the liver, kidney, and small intestine. It is characterized by an optimal *p*H of 6.5 and its extreme instability and insolubility; it appears to be bound to the microsomes. Weber and Cantero (*9*) have studied the activity of this enzyme in normal, malignant,

[1] Received for publication January 19, 1960.
[2] Presented at the Seventh International Cancer Congress, London, England, July 7, 1958.
[3] Polish Academy of Science, Department of Experimental Oncology, Institute of Immunology and Experimental Therapy, and Department of Pathologic Anatomy, School of Medicine.
[4] Third Medical Clinic, School of Medicine.

and precancerous tissues. They observed lower activity of glucose-6-phosphatase in livers of mice bearing Adenocarcinoma E0771 and a lack of activity in homogenates of Sarcoma 37, Adenocarcinoma E0771, Lymphosarcoma 6C3HED, and Novikoff hepatoma. Progressive diminution of glucose-6-phosphatase activity was observed in the liver of rats fed 4-dimethylaminoazobenzene;[5] in developing hepatomas it disappeared almost completely. The authors emphasized the absence of this enzyme from Novikoff hepatoma, which is known to originate from liver cells and not from the biliary ducts.

MATERIALS AND METHODS

The tissues used in these studies were: liver from normal C57BL mice, transplants of chrysoidin-induced hepatoma from mice of the same strain, Crocker sarcoma 180, a spontaneous mammary adenocarcinoma of mice, one primary hepatoma from rats fed 4-dimethylaminoazobenzene, and 3 transplanted hamster hepatomas. (The hamsters were obtained through the courtesy of Dr. Alexander Haddow, Director, The Chester Beatty Research Institute, London, England.)

The animals were killed by decapitation. The livers or tumors were removed immediately and placed in ice-cold saline. The tissue was then cut in thin slices, dried on filter paper, quickly weighed on a torsion balance, and homogenized with isotonic NaCl for 2 minutes in a Potter glass homogenizer. Ice was used for cooling throughout the procedure. The 5 percent homogenate thus prepared was used for determination of glucose-6-phosphatase activity by the method of Swanson (7). A maleate buffer, *p*H 6.5, and a 0.1 M solution of glucose-6-phosphate adjusted to *p*H 6.5 were used. Incubation time was 15 minutes at 37° C. Phosphate was determined by the method of Fiske and Subbarow. Protein was determined by a micro-Kjeldahl procedure. All determinations were carried out on two parallel samples.

RESULTS AND DISCUSSION

The results are summarized in table 1.

The data reveal that the chrysoidin-induced hepatoma exhibits considerable glucose-6-phosphatase activity. This activity, calculated per unit of weight, is smaller than that in livers of normal C57BL mice, but the difference is not statistically significant. When calculated per mg. of protein, the activity of glucose-6-phosphatase is identical in both hepatoma and liver because the protein content of the hepatoma is lower than that of liver. The results obtained for glucose-6-phosphatase activity in

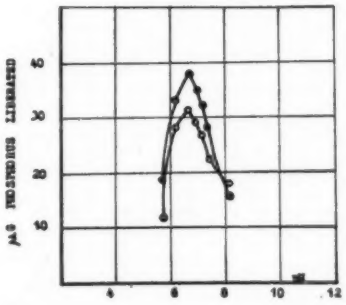
[5] *Chemical Abstracts'* nomenclature: *N,N*-dimethyl-*p*-phenylazoaniline.

TABLE 1.—Rate of enzymatic hydrolysis of glucose-6-phosphate by 5 percent homogenates of liver and tumor tissues

Tissue	Number determinations	Phosphorus liberated per 15 minutes per gm. tissue (*mg.*)	Phosphorus liberated per 15 minutes per mg. protein (μM)
Normal liver	30	7. 68 ± 0. 19*	1. 49 ± 0. 012*
Chrysoidin-induced hepatoma	22	6. 55 ± 0. 38*	1. 51 ± 0. 08*
Crocker sarcoma 180	3	0. 7	0. 23
Spontaneous mouse mammary adenocarcinoma	3	1. 3	0. 32
Primary rat carcinoma induced by 4-dimethylaminoazobenzene	1	0. 8	0. 2
Hamster hepatoma	3	0. 0	0. 0

*Figures represent mean ± standard error.

hamster hepatoma, Crocker sarcoma 180, a spontaneous mouse mammary adenocarcinoma, and primary rat liver carcinoma induced by 4-dimethylaminoazobenzene confirm the findings of Weber and Cantero. Dephosphorylation of glucose-6-phosphate in homogenates of the last three tumors is negligible as compared with liver homogenates; it may be caused by nonspecific phosphatases present in these tissues. The enzyme found in the chrysoidin-induced hepatoma seems to be identical with that present in liver. The enzymes from both sources have an optimal *p*H of 6.5 (*see* text-fig. 1). Glucose-6-phosphatase from both sources is equally labile. Upon storage in the refrigerator, both show the same rate of loss of enzyme activity.



TEXT-FIGURE 1.—The *p*H activity curves of glucose-6-phosphatase in liver homogenates (*solid circles*) and in homogenates of chrysoidin-induced hepatoma (*open circles*).

The results indicate that the chrysoidin-induced hepatoma, as distinct from other malignant tumors, contains glucose-6-phosphatase with activity and properties similar to those of the enzyme found in liver.

The lack of glucose-6-phosphatase in such neoplasms as Crocker sarcoma 180, spontaneous mouse mammary adenocarcinoma, and Lymphosarcoma 6C3HED is not surprising since the tissues from which these tumors originate do not contain this enzyme. What is striking is the findings for hepatomas from different sources; while Novikoff hepatoma,

hamster hepatoma, and primary rat liver carcinoma induced by 4-dimethylaminoazobenzene do not contain this enzyme, we found it to be present in chrysoidin-induced hepatoma at the same level of activity as in normal liver.

The presence of glucose-6-phosphatase in chrysoidin-induced hepatoma may be regarded as evidence that this tumor developed from the liver cells and not from the cells of the biliary ducts, where the enzyme is lacking. It also indicates that this hepatoma retains some features, including enzymatic differentiation, of the organ of origin.

According to Burk (*10*), the essential enzymatic reaction that governs tumor glycolysis is the phosphorylation of glucose to glucose-6-phosphate in the hexokinase reaction. Burk believes that the chemotherapy of tumors should be based on the introduction of substances which would prevent tumor glycolysis by inhibiting the hexokinase reaction. The presence of a glucose-6-phosphate-dephosphorylating enzyme in chrysoidin-induced hepatoma would have an over-all effect similar to that of inhibition of the hexokinase reaction, thus exerting an indirect influence on glycolysis and neoplastic growth. This might be one reason underlying the slow growth of our hepatoma.

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Presence and Distribution of Free Porphyrins in Transplants of a Chrysoidin-Induced Mouse Hepatoma [1, 2]

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SUMMARY

The distribution of porphyrins in the hepatoma induced by Albert with chrysoidin is described. Porphyrins were regularly demonstrated by fluorescence microscopy in parts of the cytoplasm and nucleus of the primary transplant and in metastases. It is suggested that porphyrins are of endogenous origin and that they may be related to synthesis or disintegration of the respiratory ferments which contain iron porphyrin compounds.—J. Nat. Cancer Inst. 25: 465–475, 1960.

THE PRESENCE or absence of detectable porphyrins in tumors depends on the metabolism of the neoplastic cell and in particular on disturbances of the respiratory enzymes. There are two main trends in the investigations of porphyrins in neoplastic diseases. The first is concerned with the presence of porphyrins in the tumor tissue itself and the second, with systemic disturbances of porphyrin metabolism that are directly or indirectly connected with the presence and development of the neoplastic process (*1*).

Disturbances of porphyrin metabolism result in the appearance of abnormal porphyrins in the urine. In man, no clear-cut relationship has been detected between the content of porphyrins and the incidence of tumors (*2–4*). Animals with transplantable tumors exhibited an increase of protoporphyrin in the erythrocytes (*5*) and liver, together with an enhanced urinary excretion of coproporphyrins. This phenomenon has been found in rats with a transplanted sarcoma and in rats injected with toxohormones obtained from the same tumor (*6*) and may be due to a diminution of cytochromes in the tumor or a decrease in catalase activity of the liver in animals with a neoplastic growth (*7, 8*). Vannotti (*9*) felt that synthesis and disintegration not only of hemoglobin but also of other pigments and cellular ferments containing hemins may account for the formation of small amounts of porphyrins. Greenstein (*8*) believed that during the formation of the growth, or in the growth itself, an antagonistic

[1] Received for publication January 19, 1960.
[2] Presented at the Seventh International Cancer Congress, London, England, July 7, 1958.

factor exists that affects the synthesis of iron porphyrins, which are a prosthetic group of the biologically active proteins.

It is not clear whether the porphyrins are formed in the neoplastic tissue itself. When porphyrins are administrated parenterally to humans and animals, they may be selectively localized in the neoplastic tissue (*10*) in the same way that embryonic, lymphatic, and traumatized tissues selectively concentrate exogenous porphyrins (*2, 11–13*).

Some investigators observed a red fluorescence in the neoplasms of humans and animals that had not received porphyrins (*2, 14, 15*). The prevailing opinion is that the presence of porphyrins in tumors is related to secondary processes, such as necrosis and degeneration. Bingold, Stich, and Cramer (*16*), after examining 31 human neoplasms by Fikentsher's method, came to the conclusion that a low level of porphyrins is characteristic of neoplastic tissue, which in their opinion was due to cytochrome *c* and catalase deficiency. In three cases these authors demonstrated porphyrins in liver metastases, which in their opinion was the result of a special role of the liver to porphyrin metabolism.

Because of the incomplete knowledge now available and the apparent divergence of opinion among other investigators, we present the results of our own observations.

MATERIALS AND METHODS

The tumor under investigation was the hepatoma induced by Albert in strain C57BL mice, by feeding chrysoidin (*17*), and then transplanted into C57BL and C57BL × RIII mice (*18*).

Subcutaneously transplanted tumors of varying size from over 100 mice and some primary liver tumors of C57BL mice obtained by the courtesy of Albert from one series of his experiments were studied. In some mice with lung metastases, the lungs were also examined.

The animals were decapitated. Immediately after they were bled, the tumors were prepared, sliced about 2 mm. thick, and fixed in 5 percent neutral formalin in dark-brown glass containers. The fixation time was 6 to 36 hours. Our general rule was to fix the material no longer than absolutely necessary so that frozen sections 7 to 8μ thick could be obtained. Our observations were made mostly on material fixed for 8 to 12 hours. Care was also taken to protect the material from light and to prepare it in the shortest possible time. Microscopical examinations were undertaken immediately after preparation. Unfixed sections of the tumors were prepared according to the Schultz-Braun method (*19*) and examined immediately after the mice were decapitated.

A Zeiss fluorescence microscope was used for observations. The wavelength range was provided by two connected Blue BG_3 filters (2 and 4 mm. thick) and a UG_1 filter (3.5 mm. thick). With the range of wavelength filtered by BG_3 filters, comprising a part of the visible spectrum

(from violet to blue), the fluorescence of the nuclei stained with trypaflavine and the red fluorescence of porphyrins were distinctly more intense. The ocular filters were selected according to the wavelength range inciting the fluorescence. After the fluorescence microscope was adjusted, it was possible to pass from ultraviolet light to phase-contrast and direct light without shifting the field of vision. From each block, unstained preparations and those stained for 2 minutes with an aqueous solution of trypaflavine (1:2,000,000) were examined. The stain masked the more delicate hues of the red-fluorescent cytoplasm because of the color contrast with green-fluorescent nuclei but provided an exact visualization of the red-fluorescent structures. The frozen preparations fixed in formalin were covered with a drop of tap water and those prepared according to the Schultz-Braun method (*19*), with a drop of physiological saline. For comparative purposes, some of the frozen sections from the same series examined under the fluorescence microscope were stained with hematoxylin and eosin or with hematoxylin alone. Tirmann-Schmelzer's method (*20*) was used for the detection of iron. The remaining material was embedded in paraffin, and preparations were also stained with hematoxylin and eosin and tested for iron by Tirmann-Schmelzer's method. Frozen preparations were far more suitable for porphyrin studies than those embedded in paraffin.

Microscopical examinations were paralleled by macroscopical studies. All surfaces of slices of fixed and unfixed tumors were examined under ultraviolet light.

RESULTS

Macroscopical Examinations

Most of the tumors failed to show red fluorescence on their surfaces. Some tumors exhibited slightly visible, hazy, reddish spots of varying size. The most striking but the most infrequently found fluorescence occurred in the form of rather sharply defined pink-red, brick-red, or even glowing red spots. These spots, which varied from the size of a poppy seed to that of a lentil, were seen more often subcapsularly than in central portions of the tumor. All stages of transition, from scarcely visible to spotty red fluorescence (fig. 1), were observed. No correlation could be established between the size of tumors and the presence, extent, and intensity of the red fluorescence.

Microscopical Examinations

In contrast to macroscopical examinations, the red fluorescence was observed in all tumors. Macroscopically visible red fluorescence was extensive and intense under the microscope.

Sometimes the red fluorescence was found only after examining as many as 8 to 15 preparations from several blocks. Its intensity and

extent varied considerably, not only from tumor to tumor but also from section to section of the same tumor. A characteristic of this fluorescence was its disappearance under the influence of ultraviolet light. The fading time ranged from seconds to minutes, depending on its intensity. In some preparations the intensity of the fluorescence was so slight and it faded so rapidly that no objective evaluation was possible. In sections with a more intense fluorescence, in spite of rapid fading it was possible to evaluate the material by moving the preparation during the observation so that comparison of irradiated and nonirradiated fields could be made. In the most intensely fluorescent preparations irregular, hazy, reddish spots of varying size appeared, but they faded away very quickly. Where the concentration of porphyrins was greatest there were red, more slowly fading, bizarre serpiginous areas, many globules, irregular spots, rings, and more slowly fading bright-red spots. It is of interest that the serpiginous areas were rarely seen without red shining spots and vice versa. Usually, the large fluorescent areas contained numerous tiny red spots. In the unstained preparations described and observed only under the fluorescence microscope, it was not possible to establish which structural tissue elements were related to the red fluorescence. This problem was almost completely solved by examining the same areas under phase-contrast and direct light and also by selecting a fluorochrome that stained nuclei.

After much experimentation with nuclear fluorochromes, trypaflavine was chosen. These methods are not perfect, but each has its advantages and the results thus obtained supplement one another.

In the color photographs are shown some details of the preparation fluorochromized with trypaflavine (Agfacolour-Negativfilm Ultra T).[3]

Because of the relatively low sensitivity of the film and of a certain masking effect of trypaflavine, these photographs reproduce, apart from green nuclei and greenish cytoplasm, only slowly fading and intensely fluorescent structures; moreover, they do not represent typical patterns because the preparations selected for photography contained particularly large amounts of porphyrins.

It could be demonstrated that the fluorescing aggregates were visible in unstained preparations. In typical patterns with a small and moderate amount of porphyrins, red-fluorescent areas occurred, as a rule, in the cell cytoplasm. Morphologically, the pattern of the red fluorescence corresponded with the foamy or granular structures, which were visible in the cytoplasm, in routinely stained paraffin preparations (fig. 2).

Fairly often there was a red halo at the periphery of "black" vacuoles—most commonly in large, polymorphous cells. Tiny, bright, red, glowing, irregular spots were seen in many but not all the cells with a red cytoplasm. In normal light the spots were colorless and appeared in greater numbers in regions with a distinctly red fluorescence. Here and there they occurred as solitary spots in cytoplasm that showed no other

[3] We wish to acknowledge the assistance with the color photography of Mgr. Bronislaw Kupiec, Head of the Photography Department of the Medical School in Wroclaw.

fluorescence. Rarer were vacuoles filled completely or only partially with red-fluorescent matter, which under phase-contrast invariably had a granular structure. This was also true occasionally in material examined under the fluorescence microscope. Some of them were faintly olive green in normal light. The most intense red fluorescence was found almost invariably in the irregular globules and specks of brownish-yellow cytoplasmic pigment, described by Albert and Orłowski (*18*). These globules partially filled the vacuoles and, occasionally, completely filled them. A few showed weak dark-red fluorescence; many, in spite of the absence of red fluorescence, were surrounded by a slowly fading bright-red halo (fig. 3); only a few exhibited neither fluorescence nor red halo. In paraffin preparations, after the solution of paraffin with xylene, the globules showed no fluorescence. After treatment with such solvents as ethyl ether or ethyl acetate acidified with glacial acetic acid, a small red halo, fading more or less rapidly according to the degree of acidity, appeared at the periphery of globules. Some fluorescence still remained even after 24-hour extraction in ethyl acetate acidified with acetic acid. This iron-free pigment corresponded in some respects to that described by Borst and Königsdörfer in a case of congenital porphyria (*21*). In some strongly fluorescent preparations the red fluorescence was also found in intranuclear inclusions (fig. 4); as in the cytoplasm the fluorescence involved only part of the intranuclear inclusions. In our material, the brown pigment described by Albert and Orłowski (*18*) was not seen.

As with transplanted tumors, the red fluorescence was found in primary chrysoidin-induced hepatomas and in some of their lung metastases.

Spectrophotometric examinations of porphyrins, which were dissolved in 25 percent HCl and extracted with ethyl acetate and glacial acetic acid from liver tissue, showed a characteristic porphyrin curve with an absorption maximum in the wavelength of 4100 Å.[4]

The extracted porphyrins were also examined repeatedly by the chromatographic method of Chu and Chu (*22*); we invariably detected only one spot with an Rf value ranging from 0.70 to 0.76. This corresponded to the Rf value of protoporphyrin containing two carboxyl groups.

DISCUSSION

Necrotic changes in the tumors were usually comparatively slight. Even large tumors degenerated and underwent ulceration slowly, and those selected for examination were covered by intact skin. Particular attention was paid to the relationship between necrotic lesions and the distribution, extent, and intensity of the red fluorescence. The amount of fluorescence was unrelated to the amount of necrosis in the tumors. The

[4] Spectrophotometric examinations were performed by Mgr. Przemysław Masztalerz, of the Biochemistry Department, Institute of Immunology and Experimental Therapy, to whom we wish to express our thanks.

fluorescence was usually more pronounced in the healthy tissue than in areas of necrosis. When present, the red fluorescence in necrotic lesions was always weak and diffuse. Although morphological changes in cells, particularly in regions showing a great accumulation of porphyrins, might be regarded as damage or as necrobiotic lesions, the mere presence of porphyrins is by no means evidence of degeneration or reduction in the vital activity of cells because the red fluorescence was observed in the cytoplasm of cells undergoing mitotic division. If there is any relationship between necrobiosis and the presence of porphyrins, the former is not an etiological factor but rather a secondary process related to the excessive accumulation of porphyrins.

Harderian glands of mice normally contain a considerable amount of porphyrins. There is little likelihood of a direct relationship between the presence of porphyrins in harderian glands and their presence in hepatoma, since repeated examinations of harderian glands revealed no correlation between the intensity of the red fluorescence of these glands and the degree and intensity of the red fluorescence in hepatoma.

This study does not supply any conclusive data about the origin of porphyrins discovered in hepatoma, but it does suggest endogenous origin. It should be stressed that Albert and Orłowski (*23*) found a normal or even slightly increased respiration rate in hepatoma tissue as compared with normal liver tissue. It is likely that there is some correlation between this phenomenon and the presence of porphyrins in the hepatoma.

Red fluorescence occurs irregularly in the tumor, suggesting a certain relationship between a particular state of the neoplastic cells of these regions and the presence of porphyrins. This fact is of interest regardless of whether porphyrins have an endogenous or exogenous origin. Even if it could be proved that porphyrins are not formed in the tumor and that its cells are only the storage site of porphyrins manufactured elsewhere or supplied in the food, their focal distribution would still not be explained. The basis of this phenomenon must be sought in the metabolism of neoplastic cells themselves, and especially in ferments that contain iron porphyrins.

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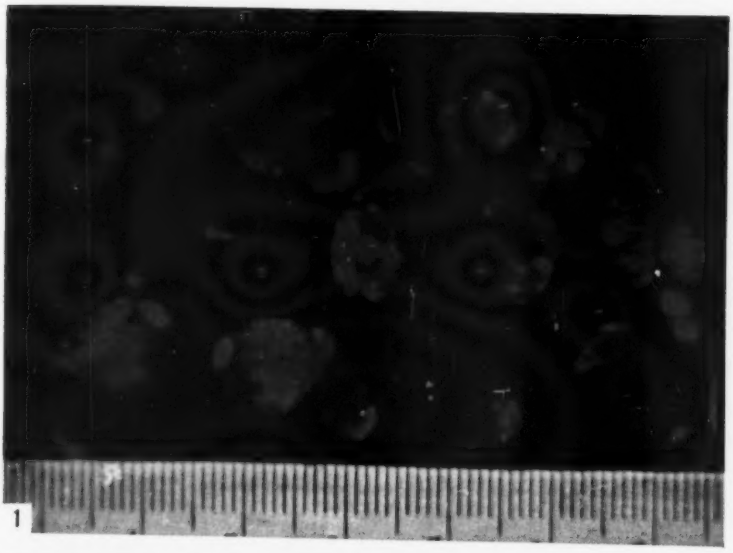
PLATE 58

FIGURE 1.—Color macrophotography of sections of some hepatomas with exceptionally extensive and intensive spotty red fluorescence.

FIGURE 2.—Rarely encountered, extensive, irregular red fluorescence of the hepatoma cytoplasm with bright-red fluorescent globules and spots. × 285



1

2

MEDRAS

PLATE 59

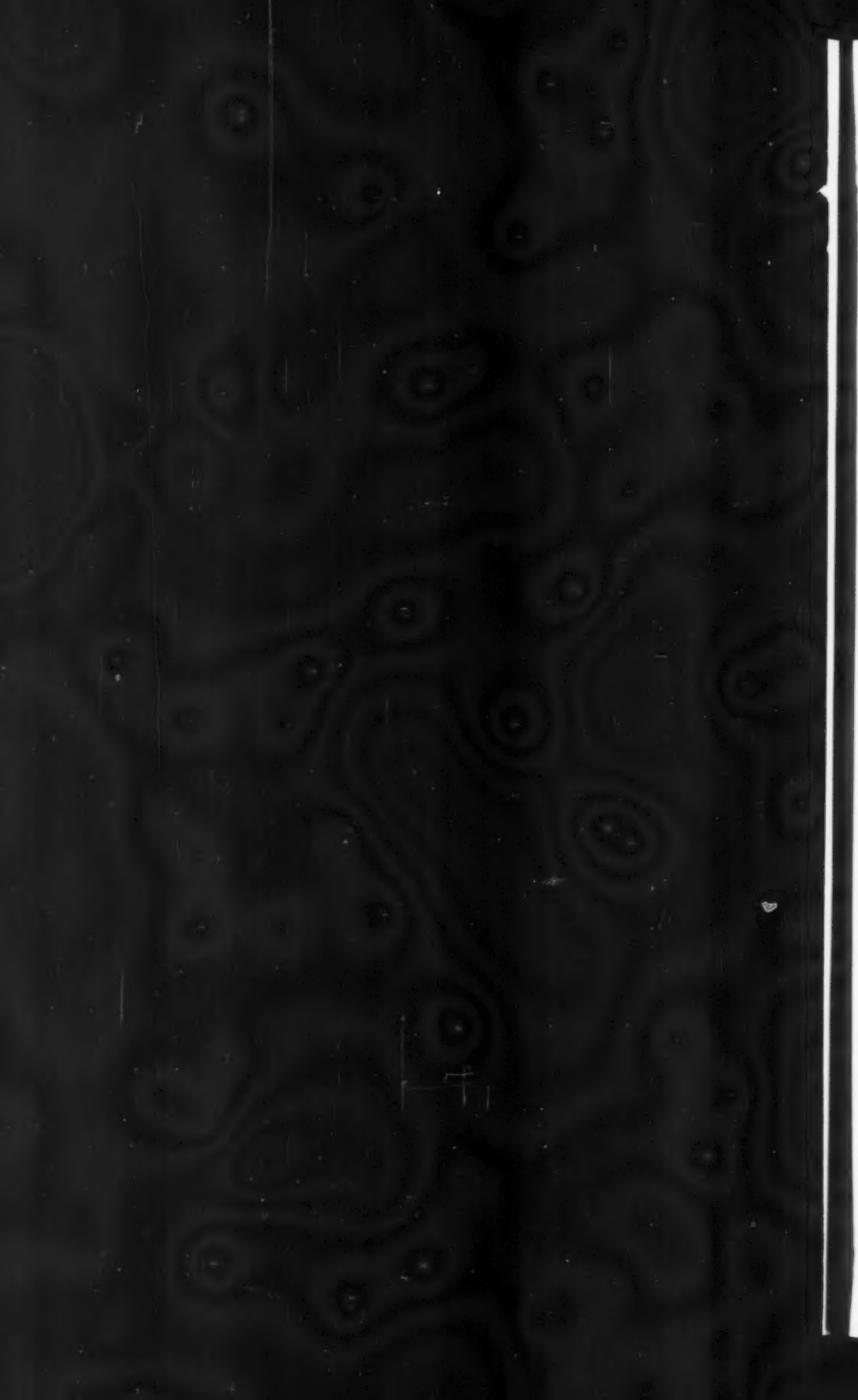
FIGURE 3.—Numerous bright-red fluorescent globules and spots. In spite of great accumulation in a small area, the cell cytoplasm is only slightly fluorescent. Globules with intensely fluorescent centers are deposits of brown pigment surrounded by a red fluorescent halo. This pigment is clearly visible in routine preparations. Hematoxylin and eosin. × 285

FIGURE 4.—Almost *center* of photograph *see* nucleus with tiny, bright-red fluorescent "nucleolus." × 285

3

4

MEDRAS



Autoradiographic Analysis of Cell Proliferation in Spontaneous Breast Cancer of C3H Mouse. I. Typical Cell Cycle and Timing of DNA Synthesis [1, 2]

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SUMMARY

Primary breast tumors of the C3H mouse and autotransplants of these tumors have been labeled *in situ* by intraperitoneal or intravenous injection of tritiated thymidine. From the frequency of labeled mitotic cells at various times after injection a typical cell cycle has been formulated. This cycle is completed in 1 to 3½ days. The synthesis of deoxyribonucleic acid (DNA) occurs in 9 to 13 hours, and there is a 1- to 4-hour period between the completion of DNA synthesis and the onset of mitosis. In many tumors this typical cycle applies to nearly all dividing cells. A comparison between different tumors and between these tumors and other cell systems indicates that variation in intermitotic time is primarily related to the duration of the presynthetic period. The growth rate of these tumors is limited by factors other than the rate at which the cells synthesize DNA.—J. Nat. Cancer Inst. 25: 477–484, 1960.

THIS IS a study of the kinetics of deoxyribonucleic acid (DNA) synthesis and the timing of the mitotic cycle in mammalian-tumor cells. The cells are labeled with a radioactive precursor of DNA and then, with autoradiography, they are scored as they pass through their next mitosis. The technique has been effectively applied to bean roots (*1*), ascites tumor cells (*2*), bone marrow cells in culture (*3*), intestinal epithelium (*4*), and a variety of other systems. This study of the breast-tumor cells of the C3H mouse represents an application of the technique to solid spontaneous tumors growing *in vivo*.

Both undisturbed primary tumors and autotransplants of primary tumors were used. Autotransplants permitted the removal of multiple samples at various intervals after labeling and avoided the anticipated variation between individual primary tumors without introducing the problems of immunological incompatibility. When the results with transplants demonstrated little variation between animals, the extension to individual primary tumors was considered worth while. Data on a large number of primary tumors gave a better indication of variations in

[1] Received for publication December 15, 1959.

[2] Supported by grant C-3896 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by the Samuel S. Fels Fund, Philadelphia, Penna.

the timing of DNA synthesis. The undisturbed tumors were effective controls for determining whether repeated operations might have a physiological effect on the tumor cells or whether autotransplants might behave differently from primary tumors.

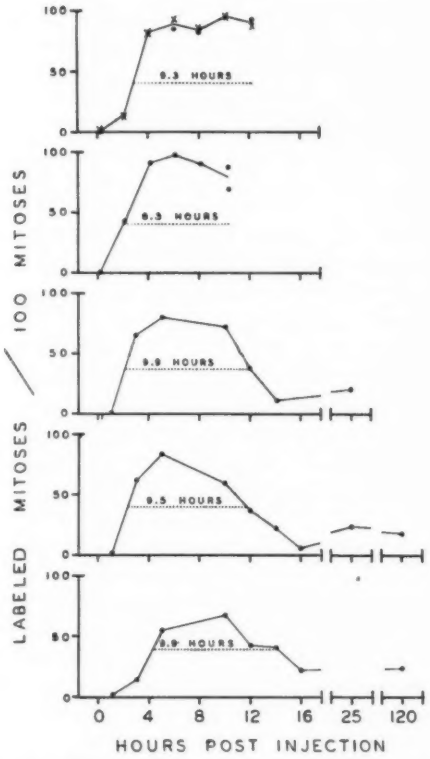
Tritiated thymidine was chosen as the labeled precursor of DNA. Thymidine has a high biochemical specificity for DNA, and tritium is a radioisotope well suited for the resolution needed to identify individually labeled cells by autoradiography.

METHODS

Autotransplants.—Female C3H/Jax mice with spontaneous breast tumors were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. A mouse with a small, readily resectable tumor was selected and anesthetized with ether. The tumor was removed by surgical procedure and a homogeneous area was isolated and cut into multiple fragments varying from 1 to 8 mm.[3] Approximately 10 fragments were placed in various parts of the subcutaneous tissue of the trunk of the anesthetized mouse. The subcutaneous space was entered through the original tumor bed or, in some cases, through a small incision on the opposite side of the trunk. Anatomical separation of the fragments was insured by positioning the implants with a trocar and by avoiding excessive loosening of the skin. The incisions were sutured and the animal was caged and fed in a routine manner. The remaining fragments were fixed and stained.

When the transplantation procedure was successful, a crop of nodules appeared on the trunk of the mouse. After 2 to 4 weeks, when the nodules had reached a diameter of 5 to 10 mm., the mouse received an injection of 80 μc. of tritiated thymidine (Schwarz Laboratories, 350 mc./mM). As soon as 13 minutes after inoculation, the mouse was anesthetized with ether and a single nodule was removed. The operation was kept as brief and bloodless as possible. The mouse was returned to its cage for varying periods until the next procedure. As many as 9 operations were performed. One of the 6 animals with successful transplants died after the second operation and the data are not included. The remaining animals withstood the repeated surgical procedures remarkably well. The times at which tumors were removed are shown in text-figure 1. Because of inaccessible tail veins, mouse D, represented by the fourth plot in the figure, was injected intraperitoneally. The other 4 animals received intravenous injections.

The tumors were fixed in neutral formalin, dehydrated in alcohol, and embedded in paraffin, with a high melting point. Sections were cut at 2 μ and mounted on clean slides. To reduce cytoplasmic basophilia and insure the extraction of all thymidine and thymidine derivatives not incorporated into DNA, the sections were hydrolyzed for 3 minutes in 1 N HCl at 60° C. The autoradiographs were prepared by a modification



TEXT-FIGURE 1.—Labeled Mitoses/100 Mitoses as a function of time in 5 mice with autotransplants of their primary breast tumors. Tritiated thymidine was injected at time zero. Each plot represents a separate animal, with animal A at *top*. The pair of symbols in the first plot represents the count of two independent observers. *Dotted* lines, drawn across at the 40 percent level indicate duration of the mitotic wave.

of the dipping method (*5*) with NTB3 bulk emulsion.[3] No protective coating was used between emulsion and section. After the slides were dipped, they were held vertically for a moment while the emulsion set, and then were placed on flat trays and allowed to dry slowly at room temperature. During the exposure period the autoradiographs were stored at 4° C. in light-proof boxes containing a desiccant. The autoradiographs were developed for 6 minutes at 10° C. in D-19 diluted 1:1 with water. After fixation and washing, they were stained with hematoxylin and eosin and were permanently mounted under coverslips. The optimal exposure time ranged from 3 to 8 days.

To insure an objective count, the slides were identified by code during the counting. Each slide was independently counted by at least two

[3] The emulsion was generously supplied by the Eastman Kodak Company.

observers, using an oil-immersion objective and a total magnification of 1,250×. The sections were scanned in a deliberate pattern, and mitotic cells were identified and recorded as either labeled or unlabeled. The distinction between labeled and unlabeled mitoses could be made with confidence, because the exposures were selected to yield an average of 20 grains per nucleus and the background was usually less than 1 grain per nucleus. In an ideal preparation, 3 separate grains over a nucleus were considered positive. Cells in most stages of mitosis were readily identified. However, the earliest stage in prophase at which a cell could be classified as mitotic varied somewhat from slide to slide because of variations in fixation, overlying silver grains, and thickness of the emulsion. The determination of Labeled Cells/100 Cells was made from counts of approximately 2,000 cells in 10 fields chosen at random.

Primary tumors.—The data on individual primary tumors come from two sources: (1) There is a variety of labeled tumors which represents controls or preliminary explorations with the methods—31 primary tumors from 22 mice are in this category. At a specified time before the mice were killed and the tumors removed, each mouse received from 50 to 100 μc. of tritiated thymidine by parenteral injection. (2) Fifteen additional mice bearing 29 tumors were chosen specifically for this study and were used to fill in time gaps left by the previous group. Each of these animals received an intravenous injection of 66 μc. of tritiated thymidine at various times before death.

The preparations were the same as for the transplanted tumors except that some slides required exposures up to 1 month. Only one observer counted many of these slides, but a minimum of 200 mitoses was counted for each tumor.

RESULTS

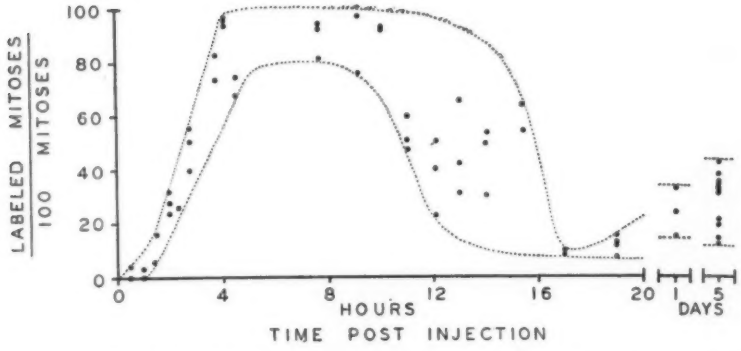
Occasional breast tumors in C3H mice show labeled cells distributed in organized patterns relative to the position of blood vessels, but most tumors are labeled randomly with the labeled cells scattered throughout the tumor. Among different primary tumors there are often differences in histology and mitotic index, and the values for the frequency of labeled cells may range from 5 to 25 percent. The variation among the 5 tumors used for the transplants was not so great, as can be seen from the compilation of Labeled Cells/100 Cells in table 1. More to the point, the transplants from any animal yield frequencies of labeled cells which remain reasonably stable throughout the experiment, apart from a tendency of the values to decrease during the period when mitoses are unlabeled and then rise progressively, coincident with the wave of labeled mitoses. The stability of these counts reflects the tendency of the transplants to resemble the parent tumor and is also an indication that tritiated thymidine is available for a short time compared to the duration of DNA synthesis. The acuteness of labeling applies after intraperitoneal as well as intravenous administration of the labeled precursor.

TABLE 1.—Frequency of labeled cells

Time (*post injection*)	Labeled Cells/100 Cells				
	A	B	C	D	E
13 minutes	14. 9	12. 0			
1 hour			15. 9	9. 1	11. 9
2 "	11. 4	14. 3			
3 "			11. 4	8. 9	8. 2
4 "	9. 1				
5 "			10. 4	8. 4	11. 9
6 "	13. 2	18. 2			
8 "	9. 3	20. 2			
10 "	17. 4	18. 6	13. 3	10. 3	10. 0
12 "	11. 7		13. 6	11. 8	16. 6
14 "			18. 0	8. 4	12. 0
16 "				11. 4	14. 5
25 "			19. 8	9. 8	
120 "				10. 7	18. 6

The mitotic data for the 5 animals with transplanted tumors are shown in text-figure 1. In the first plot, the individual values obtained by two observers are kept distinct. These are typical of the consistency of the data. In the remaining plots, the data are averaged, with each point representing a minimum of 200 mitoses. All 5 animals show similar changes of Labeled Mitoses/100 Mitoses with time. There is an initial period when few labeled mitoses are observed, followed by an abrupt rise, a plateau, and then a somewhat less abrupt fall. The latent period, presumably representing the interval between the end of DNA synthesis and the onset of recognizable mitosis, ranges from 1 to 4 hours for the typical mitotic cell. In two cases the plateaus involve about 90 percent of the mitoses, while in the others they vary between 60 and 70 percent. The experiments represented in the first 2 plots are too short to show the fall from the plateau; it can only be said that the plateaus are at least 8 and 9 hours long. Taking the width of the curve at the 40 percent level as an index of the time span of the mitotic wave, the results of the other 3 experiments group closely at slightly less than 10 hours. In two cases there is an indication that the synchronous wave is followed by a trough and then by a return to an intermediate value.

The fraction of labeled mitoses for each of the individual primary tumors is plotted in text-figure 2. This composite has the same general shape as the data represented in text-figure 1, with latent period, sharp rise, plateau, and fall to a trough. However the spread of values, particularly during the fall and at later times after loss of synchrony, is readily apparent. The primary tumors have a typical latent period of 1 to 2 hours. In 8 tumors the plateau of labeled mitoses is 92 percent or higher. The duration of the wave, with the same index as before, ranges from 9 to 13 hours, with the majority of tumors fitting closely to the 10-hour estimate obtained from the transplants. The equilibrium values at 5 days for 11 tumors range from 12 to 43 percent labeled mitoses, with a cluster of values at 35 percent.



TEXT-FIGURE 2.—Labeled Mitoses/100 Mitoses as a function of time in 51 primary tumors from 37 mice. Tritiated thymidine was injected at time zero. Each point represents a single primary breast tumor. *Dotted* lines include all the observed values.

DISCUSSION

The over-all consistency of these results, especially the similarities between transplanted and primary tumors, strongly indicates that these data reflect a general property of the spontaneous breast tumor of the C3H mouse. A typical mitotic cycle for the cells of these tumors would involve 10 hours in the synthesis of DNA and a delay of 1 to 4 hours between the end of DNA synthesis and the onset of mitosis. The duration of the cycle could be estimated by dividing the duration of DNA synthesis by the fraction of cells which are labeled, but this is valid only when all cells are contributing to cell division. The third paper in this series will show that there is a sizable fraction of nondividing tumor cells and that it is better to calculate the duration of the cycle by using, as the denominator, the fraction of mitoses which is labeled after the cells become asynchronous. Taking the mitotic data at 5 days, and with 10 hours as the duration of DNA synthesis, the duration of the cell cycle in these tumors ranges from 1 to 3½ days. Compared to this variation of over 2 days for the intermitotic time of different tumors, the interval between the onset of DNA synthesis and mitosis ranges from 11 to 16 hours, with a variation of only 5 hours. This indicates that the differences in intermitotic time in different tumors are due largely to differences in the presynthetic phase of the mitotic cycle.

Evidence for cell heterogeneity within a tumor can appear in a study of this type in two ways. If the cells were perfectly synchronous and technical difficulties did not prevent or obscure the incorporation of labels, 100 percent of the mitoses would be labeled in the plateau. Asynchrony due to lack of conformity of individual cells or small groups of cells would lower the measured height of the plateau. The second type of variation is regional in scope. It appears as a difference in the fraction of labeled mitoses found in different parts of the same tumor. Regional variation

may coincide with distinctive histological features. It is most commonly seen in tumors which have given rise to several subsidiary nodules, even when these nodules are histologically alike. Unlike asynchrony, which is best evaluated during the stable parts of the wave, regional variations are most apparent when counts of labeled mitoses are changing rapidly, particularly during the fall from the plateau. Only a small fraction of the tumors demonstrates regional variation, and the values obtained from deviant subsamples do not extend beyond the range shown in text-figure 2. With both types of variation, one cannot distinguish whether the cause is local environmental effects or the cloning out of distinctive cell lines. In most of these tumors, the dividing cells conform closely to a typical cycle.

If the cells were completely synchronized and the plateaus were at 100 percent, it would not be difficult to determine the duration of the plateau and, by inference, the duration of DNA synthesis. In the absence of such conformity, approximate limits to the plateau must be defined by an arbitrary standard. In this study, the 40 percent figure was selected before tabulation of the data. It represents a value which is greater than the prevailing frequency of labeled cells in this type of tumor and, with only one exception, also exceeds the frequency of labeling among mitoses after asynchrony. Its chief virtue is that it is roughly half the height of the plateau and most likely represents the typical or majority behavior. A higher figure would have excluded most labeled mitoses from the estimate; a lower figure might yield an arbitrary composite in which the beginning of the plateau would be defined by a small segment of cells arriving in mitosis ahead of schedule, and the end by another segment of cells proceeding at a slower rate.

The period between the completion of DNA synthesis and the onset of mitosis in these tumor cells is comparable to that of 1 hour for myelocytes in the dog (*6*), 1½ hours for intestinal crypt cells of the mouse (*4*), 2 to 3 hours for HeLa cells in culture (*7*), and 3 to 4 hours for human bone marrow cells in culture (*3*). It is somewhat shorter than the 6 hours described for ascites tumor cells in the mouse (*2*), chick fibroblasts in culture (*8*), and the cells of the broad bean root tip (*1*). In these various systems, the shortest duration of DNA synthesis is 5 hours for myelocytes in the dog and for both HeLa cells and fibroblasts in culture; the longest duration is 12 to 15 hours for human marrow cultures or ascites cells *in vivo*. Again the values obtained for the breast-tumor cells indicate that they are not unlike many other proliferating cells. A comparison of the intermitotic times indicates that the breast-tumor cells have a longer cycle than these other cell types. Bone marrow cells cycle in 45 hours, bean-root cells in 30 hours, and the remaining cell types in less than a day. In particular, ascites cells and intestinal epithelium proliferate *in vivo* much more rapidly than breast-tumor cells. Such differences are not surprising. Different tissues, including tumors, obviously have different growth rates, and growth rate is very much a function of intermitotic time in a population consisting largely of dividing cells. What is not so obvious is whether

these differences relate to the duration of DNA synthesis and the interval between synthesis and mitosis. In a breast tumor there are likely to be many factors determining the growth rate. These include the innate capacity of the cells for growth, endocrine effects, and the restraining influence of inefficient and inadequate circulation. Whatever the mechanism, the results of this study indicate that the presynthetic portion of the cell cycle is the major source of variation of intermitotic time. The remainder of the cell cycle, including the synthesis of DNA, varies by an order of magnitude less than the total cycle. If DNA synthesis is implicated at all, it is through the sequence of events initiating synthesis. Like mitosis, DNA synthesis and the postsynthetic interval may be considered as occurring by rote, once they have been triggered by some mechanism in the cell. Under other conditions such as pregnancy, or in other cells, it is conceivable that the rate of DNA synthesis becomes the limiting factor in the rate of cell proliferation; but for the conditions of these experiments, the rate-limiting factor must be elsewhere in the cell's economy.

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Autoradiographic Analysis of Cell Proliferation in Spontaneous Breast Cancer of C3H Mouse. II. Growth and Survival of Cells Labeled With Tritiated Thymidine [1, 2]

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SUMMARY

Autotransplanted breast tumors of C3H/Jax mice were removed at various periods up to 4 weeks after intravenous injection of 50 or 100 μc. of tritiated thymidine. Autoradiographs of the tumors were counted in detail and the following indexes were determined: Cells/Field, Mitoses/100 Cells, Degenerates/100 Cells, Labeled Cells/100 Cells, Labeled Mitoses/100 Mitoses, Labeled Degenerates/100 Degenerates, Labeled Mitoses/100 Labeled Cells, and Labeled Degenerates/100 Labeled Cells. The indexes for the transplants derived from any of the primary tumors are consistent with each other and are distinct from those of transplants derived from other primaries. With these indexes it is possible to compare the relative size of the labeled and unlabeled cell populations and the rates of growth and degeneration in these populations at various periods after labeling. The data indicate that growth and survival are similar in labeled and unlabeled cells and are stable with time. These results have dual significance: (1) Under the circumstances of these experiments, tritium incorporated into the deoxyribonucleic acid of labeled cells has no demonstrable effect on growth or survival, and (2) as a passive cell marker tritiated thymidine is practical and effective for studying many aspects of tumor growth. To account for the ultimate fate of labeled cells, a model is suggested in which the number of labeled chromosomes in the population remains constant after the first division post labeling. The data from one tumor conform closely to the model in that they follow the prediction that the number of labeled cells does not increase beyond the sixth generation after labeling.—J. Nat. Cancer Inst. 25: 485–500, 1960.

THE TYPICAL cell cycle of the C3H breast tumor was described in the first paper in this series (*1*). In contrast to demonstrating the kinetics of thymidine incorporation into deoxyribonucleic acid (DNA), this paper and a third explore the potentialities of tritiated thymidine as a passive cell marker. The comparative survival and proliferation of labeled and unlabeled cells are observed for several weeks after the isotope is incorporated. In the third paper, the fraction of cells in the tumor contributing to growth is estimated. The present report deals with the question of a

[1] Received for publication January 14, 1960.

[2] Supported by grant C-3896 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by the Samuel S. Fels Fund, Philadelphia, Penna.

tritium hazard to the labeled cells and the fate of the label as the cells proliferate.

The radioisotope tritium has two properties which suggest that tritiated thymidine incorporated into DNA would be much more hazardous than a variety of other tritiated compounds distributed randomly in the cell: (1) The β-particle emitted when tritium disintegrates has an average range which is less than the radius of the cell nucleus. Within this range and often within the cell nucleus from which it arises, the particle produces hundreds of ionizations. (2) When the tritium disintegrates it transmutes to helium, thus leaving the residual thymidine moiety *in situ* in DNA chemically unstable. Although these changes seem drastic, it is not clear what effect either of these processes will have. The conclusions of the few reports on toxic effects from tritiated thymidine are divided (*2–6*), and a number of observers have commented on the uncertainties involved and the lack of apparent toxicity in labeling with tritiated thymidine (*7*, *8*). Since a study of the growth and survival of labeled cells inevitably involves considering the possible toxicity of the label, this report is as much an analysis of the effects of tritiated thymidine as it is a description of some biological properties of tumor cells.

In the previous study, in which only a single mitotic cycle was considered, the relative lack of variation in the timing of DNA synthesis made it feasible to compare different primary tumors. For present purposes, the differences in intermitotic time between different primary tumors would obscure all but the most gross effects. For this reason, only mice with multiple autotransplants of their original primary tumors were used.

METHODS

Multiple subcutaneous autotransplants of spontaneous primary breast tumors of female C3H/Jax mice were prepared as in the previous experiment (*1*). Individual tumor nodules were removed during ether anesthesia at times ranging from 1 hour to 28 days after the intravenous injection of tritiated thymidine. Initially 6 mice with suitable transplants were available. One was discarded after succumbing to the third operation and another before counting because each of its nodules was grossly different histologically. The 4 animals to be described tolerated the repeated surgical procedures well. Eventually they all died from hemorrhage or anesthesia, but only after 7 or 8 successful operations. Animals A, B, and C received 50 μc. and animal D 100 μc. of tritiated thymidine with a specific activity of 350 mc. per mM. The mice weighed approximately 40 gm.

The histological procedure and the preparation of autoradiographs by the dipping method have already been described, except that these sections were not hydrolyzed in acid before exposure. A series of autoradiographs was prepared for each tumor nodule. After allowing succes-

sive doublings of the exposure time, the slides giving the best balance between adequate exposure and good resolution of the underlying cells were selected. The minimum number of grains defining a labeled nucleus was established for each slide on the basis of background and signal, and was the same for interphasic, mitotic, and degenerating cells. Under optimal conditions this was as few as 3 grains per nucleus.

The method of counting the autoradiographs was planned to include adequately large and unbiased samples of the sections. The counting field, defined by an eyepiece reticle, was 6100 μ^2 magnified 1,250×. This standard field usually contained over 100 cells and was conveniently subdivided to facilitate the counting. The section was first scanned with low magnification to locate a coordinate which traversed a representative sample of the tumor and was parallel to one of the motions of the stage. With an edge of the tumor and the selected coordinate on the optical axis, the optics were changed to oil immersion. The cells falling in the field were counted. Then the image was put out of focus and the slide was moved a random distance along the coordinate to a new area. After the microscope was refocused, the cells in the new area were counted. This procedure was repeated 15 or 20 times until approximately 2,000 cells and the full length of the section on the coordinate had been surveyed. Each field of cells was counted for labeled cells, mitoses, labeled mitoses, degenerate cells, and labeled degenerate cells. In 5 of these fields the total number of cells was determined. Then the slide was continuously scanned along parallel coordinates adjacent to the one originally chosen, and further counts were made of the mitotic and degenerating cells until a minimum of 100 of each had been classified as labeled or unlabeled. Finally, at least 200 additional labeled cells were identified and recorded as interphasic, mitotic, or degenerative.

These tumors contained little evident stroma and no gross necrosis, so that, except for occasional leukocytes, all nucleated cells were counted. Differences in fixation and in intensity of the autoradiograph led to slight differences in the first stage of prophase at which labeled cells could be identified with certainty. However every effort was made to keep the scoring of labeled and unlabeled mitoses entirely comparable on any slide and as comparable as possible among the slides. To be equally unbiased with degenerating cells, the counts were limited to a particular group of cells that could be precisely identified. Within this group, the cells had nuclei which had lost their chromatin structure and were homogeneously and densely stained with hematoxylin having a purple cast. The nuclei were sometimes fragmented into a cluster of spheres. More often they were still intact but were smaller than surrounding interphase nuclei and had altered shapes. There was a characteristic empty perinuclear space surrounded by very scant cytoplasm. These cells were still an integral part of the cellular pattern of the tumor.

The data were collected by one observer; however each entry includes at least two independent sets of cell counts. The total number of cells comprising the final count was approximately the same for the tumor

TABLE 1.—Histology and standard counts

	Time (*post injection*)	Cells / Field	Mitoses / 100 Cells	Degenerates / 100 Cells	Maximum exposure (*days*)	Histology
A	2 Hours	114	0.9	1.7	24	Macroglandular, hemorrhagic
	7.7 Hours	95	1.7	0.6	24	Acinar, well organized
	4 Days	133	1.0	0.8	30	Acinar—macroglandular, and epidermoid
	6 Days	120	1.3	1.2	38	Acinar, tending toward epidermoid
	9 Days	105	0.8	1.5	60	" " " "
	14 Days	147	1.0	1.5	149	" " " "
	28 Days	104	0.9	0.7	122	" " " "
	Mean	117	1.1	1.1		
B	2 Hours	110	0.8	1.7	24	Mixed, acini and sheets
	7.7 Hours	98	1.4	1.1	24	" " " "
	1 Day	82	0.7	0.8	30	Mixed, some acini, mostly sheets
	4 Days	101	0.9	1.1	60	" " " " "
	6 Days	170	0.8	0.5	120	Acinar
	9 Days	137	1.2	0.3	120	Mixed, acini and sheets
	14 Days	128	1.1	0.5	122	Acinar
	Mean	118	1.0	0.9		
C	2 Hours	102	1.6	0.4	24	Acinar, moderately organized
	7.7 Hours	124	1.9	0.5	24	" " "
	1 Day	124	1.4	0.6	24	" well organized
	4 Days	117	1.7	1.1	24	" moderately organized
	6 Days	105	2.5	0.9	30	" " "
	9 Days	139	1.4	1.1	120	Macroglandular
	14 Days	128	0.9	1.2	149	Acinar, well organized
	Mean	120	1.6	0.8		
D	1.4 Hours	171	0.8	1.9	12	Acinar, poorly organized, cords
	2 Days	165	1.4	1.2	12	" " " "
	4 Days	172	0.7	1.4	12	" " " "
	7 Days	176	0.7	2.2	28	" " " diffuse
	9 Days	206	0.5	0.8	50	" " " cords
	10 Days	177	0.8	2.3	50	" " " "
	10 Days	182	0.8	2.0	50	" " " "
	Mean	178	0.8	1.7		

nodules of any animal and are given as the average for each index in table 2. As a precaution against inadequately exposed slides, the duplicate counts were made on successive autoradiographs from the same series. If the incidence of labeled cells increased with exposure time, successively longer exposures were examined until the counts reached a plateau. The longest exposure time used for counting is recorded for each tumor in table 1.

RESULTS

Anatomy

The autotransplants from any animal tend to have the same appearance, both grossly and microscopically. Occasionally one nodule is different from the rest (in the one animal discarded from the study the nodules were all strikingly different). The nodules remaining *in vivo* continue to increase in size but otherwise they do not change their anatomical characteristics. The histological features of each tumor nodule are given in table 1.

Nonautoradiographic Indexes

The data for the nonautoradiographic indexes are summarized in tables 1 and 4. Table 1 suggests that, except for a fall in degenerate counts in animal B, the values are independent of time. This is confirmed by the lack of significance of time sequence in the analysis of variance. The potential stability of the indexes is emphasized by the significant differences that are apparent between the animals. Thus the transplants from animal D have a higher incidence of degenerate cells, while those from animal C have a higher mitotic index. The second line of figures at 10 days for animal D in table 1 represent counts from a pulmonary metastasis. These values are similar to those from the transplants and have been included in the calculations.

The statistical analyses summarized in tables 3 and 4 follow the methods given by Snedecor for analysis of variance with multiple classifications and Hartley's sequential method of identifying deviant means (*9*). The data were selected for these calculations by the use of two criteria: (1) The values marked with an asterisk in table 2 were omitted. As will be shown, the departure of these values from the others in the sequences can be explained by the kinetics of the labeling process. (2) For each index, the animal with the smallest number of samples determined the number of sequential entries; this greatly facilitated the analyses by making the arrays symmetrical. As a result, the labeled indexes from the lung metastasis in animal D were systematically omitted, and the end samples from some of the sequences were occasionally dropped.

Autoradiographic Indexes

The data on labeled cells are summarized in tables 2, 3, and 4. Text-figure 1 shows these results for animal A. Each index will be described

TABLE 2.—Autoradiographic counts

		Time (*post injection*)	Labeled Cells	Labeled Mitoses	Labeled Degenerates	Labeled Mitoses	Labeled Degenerates
			100 Cells	100 Mitoses	100 Degenerates	100 Labeled Cells	100 Labeled Cells
A	Nodule	2 Hours	7. 4	32*	1. 5*	3. 7*	0. 7
	Nodule	7. 7 Hours	6. 9	82*	6. 0*	16. 1*	0. 7
	Nodule	4 Days	8. 8	17	15	1. 0	1. 4
	Nodule	6 Days	10. 6	14	17	1. 3	1. 7
	Nodule	9 Days	11. 7	17	18	1. 4	1. 1
	Nodule	14 Days	10. 9	13	16	1. 0	1. 7
	Nodule	28 Days	1. 1*	0*	0*	1. 2	1. 2
	Mean*		9. 4	15. 2	16. 4	1. 2	1. 2
	Mean of total cells counted per value		3, 320	210	190	1, 230	1, 230
B	Nodule	2 Hours	10. 3	28*	19	1. 6*	0. 7
	Nodule	7. 7 Hours	13. 4	93*	8. 4	17*	0. 6
	Nodule	1 Day	13. 5	33	8. 0	1. 8	0. 4
	Nodule	4 Days	13. 5	25	14	1. 6	1. 6
	Nodule	6 Days	8. 5	12	13	1. 7	0. 7
	Nodule	9 Days	6. 9	10	9. 3	1. 3	0. 6
	Nodule	14 Days	1. 4*	0*	0*	1. 1	0. 9
	Mean*		11. 0	20	12. 0	1. 5	0. 8
	Mean of total cells counted per value		3, 700	250	210	1, 040	990
C	Nodule	2 Hours	16. 4	24*	17	2. 8*	0. 6
	Nodule	7. 7 Hours	15. 1	95*	17	13*	0. 2
	Nodule	1 Day	19. 0	24	25	2. 2	0. 9
	Nodule	4 Days	13. 8	27	20	1. 5	0. 6
	Nodule	6 Days	13. 7	19	22	1. 9	1. 0
	Nodule	9 Days	16. 3	26	17	1. 9	1. 2
	Nodule	14 Days	14. 1	17	22	1. 1	1. 2
	Mean*		15. 5	23	20	1. 7	0. 8
	Mean of total cells counted per value		3, 980	330	180	1, 220	1, 050

D	Nodule	1. 4 Hours	10. 2	5. 5*	5. 4*	0. 6*	1. 3
	Nodule	2 Days	10. 7	19	13	3. 0	1. 7
	Nodule	4 Days	11. 0	17	16	0. 7	3. 5
	Nodule	7 Days	12. 9	23	21	1. 2	2. 0
	Nodule	9 Days	12. 3	19	15	0. 6	0. 8
	Nodule	10 Days	14. 6	18	7. 9	1. 0	1. 4
	Lung met.	10 Days	10. 3	12	17	1. 0	4. 6
	Mean*		11. 7	18	15. 0	1. 2	2. 2
	Mean of total cells counted per value		5, 300	230	300	620	620

* The mean does not include figures marked with asterisk.

TABLE 3.—Analysis of variance of Labeled Cells/100 Cells

Sequence	Animals				Mean
	A	B	C	D	
1	7.4	10.3	16.4	10.2	11.1
2	6.9	13.4	15.1	10.7	11.5
3	8.8	13.5	19.0	11.0	13.1
4	10.6	13.5	13.8	12.9	12.7
5	11.7	8.5	13.7	12.3	11.6
6	10.9	6.9	16.3	14.6	12.2
Mean	9.4	11.0	15.7	12.0	

Source of variation	Degrees of freedom	Sum of squares	Mean square
Time sequence	5	11.83	2.366
Animals	3	129.77	43.257
Discrepancy	15	82.85	5.523
Total	23	224.45	

$F_{time} = 2.366/5.523 = 0.43$ ($F_{0.05} = 2.90$).
$F_{animal} = 43.257/5.523 = 7.83$ ($F_{0.01} = 5.42$).

separately, but taken together, the autoradiographic data give an additional confirmation that differences between animals can be discriminated by pooling the transplants of each animal. Paralleling the nonautoradiographic indexes, animal D has a significantly higher incidence of degenerates among its labeled cells than the other animals. Animal C has a higher proportion of labeled cells than animals A, B, or D.

Labeled Cells/100 Cells

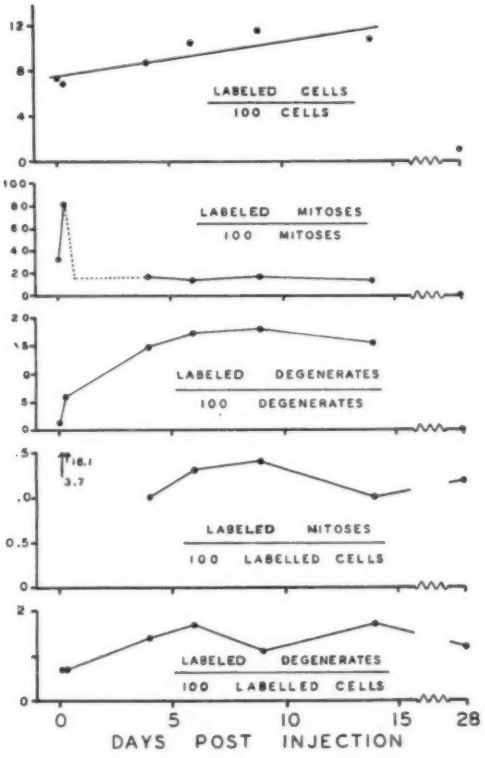
Initially this ratio represents the fraction of cells in DNA synthesis at time zero. During the period when the mitotic timing of the labeled cells is synchronous, there may be some fluctuation in the ratio. But after 1 or 2 generations, Labeled Cells/100 Cells become a good index of the relative size of the populations of labeled and unlabeled cells, with a stable value indicating that neither population has an advantage. The lack of a significant sequential trend for this index, when the 4 animals are pooled, is indicated by the analysis in table 3 and can be visualized by comparing the group means in the final column of this table. However examination of the data for individual animals shows the situation to be somewhat more complex. Animals D and C give results which are stable with time, judged by regression analyses which yield slopes 0.240 and −0.038, and which do not depart significantly from zero. However animal B shows a striking and progressive decrease with time in the proportion of labeled cells, and animal A shows a slow but steady increase. The regression line for animal A is given in text-figure 1. It has a slope of 0.322 with a 95 percent confidence limit which extends to 0.07 but does not include zero.

Labeled Mitoses/100 Mitoses

The typical cell cycle for the C3H breast tumor includes 9 to 13 hours for DNA synthesis and 1 to 4 hours between the end of synthesis and

TABLE 4.—Summary of analyses of variance

Index	Variations due to time sequence			Variations due to animals		
	F	P	Deviant means	F	P	Deviant means
Cells/Field	1.46	>0.05	None	18.4	<0.01	D>A, B, and C
Mitoses/100 Cells	2.18	>0.05	None	9.23	<0.01	C>A, B, and D
Degenerates/100 Cells	1.20	>0.05	None	5.48	<0.01	D>A, B, and C
Labeled Cells/100 Cells	0.43	>0.05	None	7.83	<0.01	C>A, B, and D
Labeled Mitoses/100 Mitoses	0.90	>0.05	None	1.50	>0.05	None
Labeled Degenerates/100 Degenerates	3.86	>0.05	None	6.99	<0.01	C>A, B, and D A and D>B
Labeled Mitoses/100 Labeled Cells	2.14	>0.05	None	1.14	>0.05	None
Labeled Degenerates/100 Labeled Cells	1.56	>0.05	None	5.98	<0.01	D>A, B, and C



TEXT-FIGURE 1.—Autoradiographic indexes from animal A plotted against time. The tumors represented by the individual points on each graph are autotransplants of a single primary breast tumor. Regression line shown in the first graph is based on the data obtained in the first 2 weeks (*see* text). After the fluctuations due to synchrony in the first division after labeling, the indexes stabilize. At 4 weeks the incidence of the 3 categories of labeled cells decreases markedly, while the indexes within the labeled populations remain stable. As indicated, this behavior is consistent with a model based on chromosomal labeling.

the onset of mitosis (*1*). The values for Labeled Mitoses/100 Mitoses for the 1- to 2-hour and the 7-hour samples, shown in table 2, are in agreement with this cycle. Although the subsequent values show no evidence of residual synchrony, they are widely spaced and do not completely exclude the possibility of secondary or tertiary waves of synchronized mitoses. When the cells become asynchronous, the values for Labeled Mitoses/100 Mitoses reflect the relative size of the labeled and unlabeled populations of cells, as well as the intermitotic time and the duration of mitosis in each population. Because of this complex of factors, unstable values such as those from animal B are very difficult to evaluate. In contrast, the stable values for animals A, C, and D, reinforced by the constancy of the mitotic index and the lack of change in the relative sizes of the

labeled and unlabeled populations, indicate clearly that the labeled and unlabeled cells proliferate at equivalent and constant rates throughout the first 2 weeks of the experiment. The 28-day value for animal A and the data from animal B will be considered in the "Discussion."

Labeled Degenerates/100 Degenerates

This index gives some indication of the relative rates of cell death in the labeled and unlabeled cell populations. It, too, is a complex of factors, including the duration of visible stages of degeneration and the relative sizes of the labeled and unlabeled populations. In addition, it has the limitation that only a particular type of cell death is represented. In animals B and C, the values of Labeled Degenerates/100 Degenerates are stable from the first samples at 2 hours, which suggests a rapid sequence between thymidine incorporation and the morphological appearance of degeneration. In animals A and D, there appears to be a 24-hour period before the ratios stabilize. In all 4 animals the ratios then remain stable throughout the first 2 weeks and are consistent with the interpretation that cell death is parallel in the labeled and unlabeled cell populations.

Labeled Mitoses or Labeled Degenerates/100 Labeled Cells

An unequivocal interpretation of the autoradiographic data already presented requires the premise that there is a fixed and preferably a one-to-one correspondence between cells which have incorporated label and those identified as labeled cells. This stringent requirement is largely avoided when the indexes are interpreted with labeled constituents in both the numerator and the denominator. As shown in table 2, the counts of Labeled Mitoses or Labeled Degenerates/100 Labeled Cells are stable throughout the series, including animal B and the sample from animal A at 28 days. Thus the growth and death rates of the labeled cells are stable, with time, and are similar in magnitude to the rates for the total population. Furthermore, the agreement among the autoradiographic, the mixed, and the nonautoradiographic indexes confirms the reliability of the autoradiographic method for detection of labeled cells.

DISCUSSION

Although Cramer pointed out the advantages of autotransplantation n 1934 (*10*), preference is usually given to the use of well-established transplantable tumors in inbred lines. The isologous transplant is a convenient and stable preparation, but it is still vulnerable to the possible effects of foreign cells and the uncertain relevance of a derived tumor. Another alternative for an experiment on multiple samples of tumor would be the surgical removal of fragments of a primary tumor. The drawback is the likelihood that previous biopsies would upset the local conditions in the tumor and compromise later samples. Multiple auto-

transplantation of primary tumors avoids the entire question of transferring cells from one animal to another and still permits compound experiments on undisturbed tumor nodules.

Beyond their relevance to the general issue of neoplastic growth, tumors are particularly suited to the study of dynamics of cell growth and survival. Unlike other proliferating tissues in the adult, tumors are not in equilibrium and need not continually discard their cells. However they have an inherent inconstancy which may be a major disadvantage. As the results of this study indicate, each primary tumor may have distinguishing characterististics and is best considered individually. In addition, there is the likelihood that inconstancy within each tumor will be manifest as cellular variation, regional variations, or instability with time.

The inconstancy of individual cells is probably the reason for the rapid loss of synchrony of labeled mitoses. A similar manifestation of cellular variation is the increasing variation in the number of grains per labeled cell observed in the later samples of tumor. With one possible exception which is relevant to the third paper in this series, this type of variation does not bias the estimates obtained from large numbers of cells. Of course the derived figures refer to a hypothetical average or, in some cases, to a typical cell, but they are still valid parameters.

Unlike the slight regional variations in the timing of DNA synthesis (*1*), two adjacent areas of tumor frequently have markedly different mitotic indexes and fractions of labeled cells. Occasionally labeled tumors contain islands of rapidly dividing cells expanding into an area with little or no manifest growth. Shortly after injection such an island is intensely labeled, but when this growth pattern is observed in tumors several weeks after injection, the expanding population is unlabeled and appears to be displacing the surrounding population of labeled, nondividing cells. This is an extreme example but some degree of regional variation is always present in these tumors. The effects of regional variation on the results were kept to a minimum by counting large samples of cells and by attempting to keep the samples representative of the entire tumor.

One determinant of regional variation in the tumors is the adequacy of regional circulation. In addition to affecting the behavior of the cells, blood supply determines the distribution of tritiated thymidine, and the situation may arise in which cells in DNA synthesis at the time of injection remain unlabeled because they have no access to tritiated thymidine. To the extent that proliferation is dependent on adequate circulation, it seems more likely that the autoradiograph gives a true indication of DNA synthesis. This is confirmed by the observation that mitoses are rarely found in the midst of unlabeled cells. A less extreme effect of the variable distribution of tritiated thymidine would be a reduction in the intensity of labeling in certain regions. This would be difficult to evaluate in tumor sections because of the many other factors which also influence the yield of grains in the autoradiograph, but the prolongation of exposure of the autoradiographs until the indexes stabilize is a reasonable precaution against bias from this source.

Instability, or inconstancy in terms of time, might occur under a number of circumstances. At one extreme would be a progressive slowing of the growth rate as the tumor increases in size. At the other, there is the possibility that more malignant variants might arise during the experiment. Since such changes in growth rate would reflect in the indexes and prevent a comparison between tumors, this is potentially the most bothersome type of variation. Fortunately, there is no indication of temporal instability in the data within the time span of these studies.

The experiments of Taylor, Woods, and Hughes (*11*) indicate that all the chromosomes become labeled in cells exposed to tritiated thymidine during the period of DNA synthesis. Furthermore, if the labeled precursor is then removed, the newly synthesized chromosomal components observed in the second division are found to be unlabeled and morphologically distinct. Although probably complicated by chromosomal exchange, this situation in its simplest form is equivalent to stating that the number of labeled chromosomes in the population remains constant after the generation in which the label is introduced. If this extension of the model beyond the first 2 generations is correct, each generation of labeled cells has half as many labeled chromosomes per cell as the preceding generation. For a mouse with its normal complement of 40 chromosomes, multiplication through the sixth generation after labeling distributes 80 labeled chromosomes to 64 daughter cells, which ends with an average of slightly more than 1 labeled chromosome per cell. Up to this point the growth of the clone can be followed by counting the labeled cells; beyond this point the labeled chromosomes will segregate in the daughter cells on an all-or-nothing basis. As the growth of the clone continues, the number of labeled cells will not increase and the fraction of labeled cells will inevitably decrease. Although they no longer reflect the growth rate of the clone, the labeled cells will still be suitable for estimates of the indexes of mitosis and degeneration.

Sister-chromatid exchange effectively increases the number of labeled units in the population. If these subchromosomal units are above the detection threshold, the effect on the model will be simply to prolong the number of generations through which the labeled cells increase. For instance, the occurrence of three random-sister-chromatid exchanges per cell per generation increases the labeled chromosomes sufficiently to average 1 labeled chromosome per cell in the seventh generation after labeling. Similarly, 8 exchanges per cell per generation extend the range to 8 generations, and 20 exchanges per cell per generation extend it to 15 generations. Even if the exchange rate in mammalian somatic cells were known, the effects of these phenomena on the fate of labeled cells would be difficult to predict without a more exact knowledge of the sensitivity of the autoradiograph.

The resolution, range, and sensitivity necessary to follow labeled cells through 6 generations are probably provided by the combination of tritium as the label and autoradiography with either dipping or stripping film as the detector. In the 1.4-hour tumor from animal D, an 8-day exposure

yields an average of 27 grains per labeled nucleus. Under favorable circumstances, this exposure can be effectively prolonged by a factor of 20, and the number of grains per nucleus necessary to detect the signal can be reduced by a factor of 5. The resulting sensitivity allows a range of 100× and is probably adequate for the detection of cells with only 1 labeled chromosome. If the cells conform to the simple model, this range will be adequate to follow the labeled cells for unlimited generations. If there is extensive chromatid exchange, the labeled units partition into subdetectable units and the labeled cells will apparently disappear as the units segregate into new daughter cells. With the particular conditions of this experiment, the fortuitous correspondence of the limit of sensitivity with the order of magnitude of single-labeled chromosomes means that both high and low incidences of chromatid exchange result in a reduced frequency of labeled cells in the sixth or seventh generation. The important difference between these two possible modes of chromosome behavior is the almost complete loss of apparently labeled cells when the chromosomal unit of label is not kept intact. After extensive chromatid exchange, the only residue of labeled cells several generations beyond the sixth would be stragglers with a long generation time or the small fraction of cells in which the remaining labeled chromosome happened to avoid exchange.

An additional consideration which bears on the evaluation of the model is the possible toxicity of tritium. In 3 of the 4 animals studied, the various indexes which compare labeled and unlabeled cells indicate that the survivals and multiplications of the labeled cells are not compromised during the first 2 weeks after labeling. In the fourth animal, B, a progressive decrease in labeled cells is apparent and the situation is more complex. Since the counts of labeled degenerates in this animal give no indication that loss of label is secondary to cell destruction, there remain 3 possible explanations: (1) The label is diluted to subdetectable levels; (2) the label reaches the limit of dilution and then segregates in daughter cells on an all-or-nothing basis; and (3) the labeled cells have a reduced rate of multiplication. Of the 4 animals, B had the fewest grains per labeled cell in the early samples. In addition, the intensity of signal decreased rapidly in the later samples, and the data in this one series leave some doubt as to whether the longest exposures reached the plateau for yield of labeled cells as a function of exposure time. These observations favor dilution as the most likely explanation, but they are not conclusive. The most decisive evidence comes from the data which were not vulnerable to dilution. This was the counts of Labeled Mitoses or Labeled Degenerates/100 Labeled Cells. The stability of these indexes and their close similarity to the analogous nonautoradiographic indexes indicate that the incorporation of label did not decrease the rate of multiplication of the cells nor increase the rate of degeneration. The uncertainty remains as to the reason for the decrease in counts, with time, but it is clear that like the others this animal shows an absence of observable toxicity from tritiated thymidine.

As to the correspondence between the model of chromosomal labeling and the data at hand, the figures for animal A are particularly relevant. The data in tables 1 and 2 will be used in the next paper to shown that the proliferating cells in the tumors of animal A have an average cell cycle of 70 hours. This means the cells will pass through 4 or 5 generations in 14 days, and 9 or 10 generations in 28 days. At 14 days the model and the data are in close agreement since the labeled cells reflect the growth of the population in both cases. But the crucial point is what happens after the sixth generation when the model predicts that in the absence of chromatid exchange the labeled population will no longer change.

In the model, Labeled Cells/100 Cells would fall to $\frac{1}{8}$ and $\frac{1}{13}$ its previous value by the ninth and tenth generations, respectively; however the remaining labeled cells should have the same mitotic and degenerative index as the labeled cells in earlier generations. In fact, the tumor removed at 28 days had a value of Labeled Cells/100 Cells which was $\frac{1}{8}$ of the previous values; and the values for Labeled Mitoses or Labeled Degenerates/100 Labeled Cells were similar to the analogous indexes for unlabeled cells and were identical to the average values for labeled cells in the earlier tumor samples. If the sparsity of labeled cells in the tumor at 28 days is attributed to loss of recognition due to dilution of the isotope, the data would be heavily biased toward the selection of slowly growing cells in the later samples, and a progressive fall in the mitotic index of the labeled population would be expected. The lack of this finding and the closeness of fit of the results with theory point to the validity of the chromosomal model of isotope dilution. An incidence of about 3 chromatid exchanges per cell per generation would still be consistent with the data at hand. However, higher rates of exchange are contradicted by these results.

In this study the incorporation of tritiated thymidine into the nuclei of tumor cells has no demonstrable effect on growth and survival *in vivo* for periods up to 2 and possibly 4 weeks. It must be emphasized that this conclusion should not be extended beyond these particular conditions. Cells may vary in their vulnerability to irradiation and particularly to tritiated thymidine, and it seems likely that at some point larger quantities of tritiated thymidine would eventually show an effect. The recent report of Johnson and Cronkite demonstrated significant toxic effects on mouse spermatogonia after a dose of 5 μc. of tritiated thymidine per gm. of mouse (*6*). However, their data were only suggestive at 1 μc. per gm. When comparing this to the lack of effect of 1 to 3 μc. per gm. used in the present study, it is important to realize that the spermatogonia are an exceptionally radiosensitive system. Experiments *in vitro* are difficult to compare with studies *in vivo* in terms of isotope dosage, but a crude comparison can be made by using the yield of grains in labeled cells. Drew and Painter reported that a 1-hour exposure to 0.02 μc. of tritiated thymidine per ml. of medium resulted in 44 grains per labeled HeLa cell in autoradiographs exposed for 24 hours (*5*). Under these conditions, this was the lowest concentration of tritiated thymidine that reduced the number of viable colonies. The autoradiographic signal

from these HeLa cells is at least an order of magnitude greater than the highest grain yield obtained from the mouse tumors (27 grains per labeled cell in an 8-day autoradiograph from the first tumor of animal D). Wimber has studied the toxicity of tritiated thymidine by measuring the yield of chromosome breaks in *Tradescantia paludosa* as a function of average grains per dividing cell in a 24-hour autoradiograph (*3*). The lowest point he has scored represents 18 grains per cell, whereas the highest score from the mouse tumors would be 3 grains per cell on his scale. Although 3 grains per cell corresponds to roughly 6 breaks per 100 anaphases on Wimber's graph, the extrapolation to the nearest measured point is far too great to warrant the conclusion that this represents a significantly toxic load of tritium.

In spite of the many uncertainties, there is some comfort in the correspondence of these three examples of tritium toxicity at roughly equivalent isotope loads, as well as the presently reported lack of toxicity at a somewhat smaller tritium load. The significance of tritium incorporated in the chromosomes may be controversial to the theoretician, but these early results suggest that it can be readily resolved by direct experiment. It is already abundantly clear that there is a wide margin between the quantities of tritiated thymidine which produce observable toxicity and the amounts needed for simple tracer studies. Nevertheless, our understanding of these toxic effects is presently limited, and cautious use of tritiated thymidine as a tracer is still recommended.

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Electron Microscopic Examination of the MC1M Tumor. I. The Tumor in Ascites Form [1]

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SUMMARY

An electron microscopic investigation of the MC1M ascites tumor is described, which shows good agreement with observations of other investigators. The cells were centrifuged, fixed with osmium, embedded in methacrylate, and sections cut for electron microscopy. The same organelles are seen in tumor cells as in all other cells, and there were no peculiarities characteristic of ascites tumor cells separating them from cells in general. Because of the intensive and abnormal metabolism of ascites tumor cells, the appearance of mitochondria received special attention. Extremely fine lamellae occurred in pairs at the same distance from each other, either concentrically around or near the nucleus or in a peripheral area of the cytoplasm in which they were also concentrically arranged around a group of mitochondria and other cell organelles. The organelles usually found in the cytoplasm were not present in areas in which these lamellae occur. The possibility that fibrils in cell cytoplasm occur in a preparatory stage of prophase is discussed.—J. Nat. Cancer Inst. 25: 501–521, 1960.

IN RECENT years, ascites tumors have developed into one of the most important requisites for experimental tumor research. Their study has supplied much important information about the genetic factors that do or do not allow transplantation and growth of tumors. The tumor cells can easily be obtained in large numbers from living animals, and under favorable conditions, can continue to live outside the organism. They are thus most suitable for studying the influence of different environments and other external factors on the growth and spread of tumors.

There are few papers on morphological examinations of such tumors, even though these tumors are readily available for treatment with current histological methods. Most of the experimentally induced tumors converted to the ascites form rapidly develop into highly anaplastic tumors and are monomorphic and undifferentiated. Studies of ascites tumor cells with the light microscope give little information about cell structure and variations caused by different external factors, mainly because the cell organelles of greatest interest, *e.g.*, the mitochondria, cannot be studied with such equipment.

[1] Received for publication February 2, 1960.

It may thus be expected that electron microscopic examinations of these tumors can give important information not only about the structure of the tumor cells but also about the variations in the different cell organelles in different environments and functional stages. Such investigations have been carried out by Selby *et al.* (*1*) with Ehrlich carcinoma and Epstein (*2*) with Sarcoma 37. Gieseking and Schümmelfeder (*3*) investigated Ehrlich carcinoma and MC1A tumors that had been transplanted subcutaneously from the ascites form and had become solid tumors. A comparison between tumors in ascites and solid forms (Sarcoma 37, and others) was reported by Dalton (*4*).

These investigations have shown that there is more of a quantitative than qualitative difference between tumor cells and normal cells. The tumor cells contain the same organelles as the normal cells, and their appearance varies only insignificantly from that of other cells in mammals and man. "The tumor cells show the same fundamental structure common to all other cells, with variations in detail such as can be found between one type of normal cell and another." (Epstein, *2*). This is, of course, most surprising when one considers that there are great biological differences.

The tumor, MC1M, originated from a connective-tissue tumor which was induced by the subcutaneous injection of methylcholanthrene in strain C3H mice and later converted to the ascites form (*5*). In this article we describe an electron microscopic investigation of the MC1M ascites tumor, which will serve as a basis for continued work on these tumors under different environmental influence and conditions of growth.

MATERIALS AND METHODS

Strain C3H mice of both sexes, weighing 20 to 25 gm., were inoculated intraperitoneally with 0.1 ml. of ascitic fluid from a mouse with MC1M ascites sarcoma. The animals were killed by fracturing the cervical vertebrae 3 or 4 days after inoculation. The abdominal wall was opened at the midline and the ascitic fluid was drawn out by means of an ordinary hypodermic syringe with a fine needle. The fluid was then transferred directly to a centrifuge tube containing a like volume (usually 1 ml.) of cold, 2 percent osmium solution, buffered to a *p*H of 7.2. After centrifuging at low speed for a short time, the mixture of fixative and ascitic fluid was poured off and replaced by 1 percent cold, buffered osmium solution in which the cells were fixed for 1 hour. The cells were well mixed with the fixative by repeated shaking of the tube or by careful stirring with a glass rod. When fixation was completed the suspension of cells was centrifuged and the fixative replaced by Tyrode's solution which was repeatedly changed within 1 hour. The cells were then dehydrated for 30 minutes each in 70 percent, 90 percent, and absolute alcohol. The alcohol was then replaced by a mixture of equal parts of absolute alcohol and methacrylate (19 parts butyl to 1 part methyl meth-

acrylate). After several quick changes of this mixture, pure methacrylate was added, together with an accelerator (benzoyl peroxide). Before each change of fluid described, the mixtures were centrifuged at low speed for a short time.

After standing in the pure methacrylate for 1 hour, portions of the sedimented cell mass from the bottom of the tube were transferred to small gelatine capsules filled with methacrylate which had been prepolymerized by heating and had become viscous. Polymerization continued for 24 hours at 57° C.

The sections were made with a glass knife partly on Sjöstrand microtome and partly on a Leitz ultramicrotome. They were viewed in an electron microscope, RCA Model 2D, with condensor and objective-aperture diameters of 0.005 and 0.003 inches, respectively.

The electron optical magnification varied between 3,500 and 20,500×. The negatives were enlarged photographically to suitable size.

OBSERVATIONS

A general view of a tumor cell is shown in figure 1. In most cases the cells are nearly round or slightly oval with a large number of fine protrusions from the surface. These have sometimes been called pseudopodia (1, 2, and others), a name which is unfortunately quite misleading as there is no evidence that the cells are capable of active movement or phagocytic engorgement of foreign bodies. The protrusions, like the cells themselves, are surrounded by a simple cell membrane. The nucleus occupies a large part of the cell volume. In the nucleus there are 2 large nucleoli—1 lying against the nuclear wall. The electron-dense substance is diffusely arranged throughout the whole nucleus. In the cytoplasm several mitochondria and other cell organelles are seen. These appear in greater detail in the illustrations that follow.

Figure 2 is a general view of another cell. Here the visible part of the nucleus occupies a much smaller part of the cell and is displaced to one side. The nucleoli are not visible in this section. In the cytoplasm there are a large number of mitochondria and several large, round, electron-dense bodies of somewhat shrunken appearance, which seem to correspond to the optically double-refracting granules visible in these cells with the light microscope (lipide granules). The right of the cell contains an area of cytoplasm which is lighter than its surroundings. This area is almost completely without mitochondria in the periphery but has many concentrated mitochondria in the center, including several large lipide granules. The same area is seen in figure 4. In other cells a similar lighter zone, almost without cell organelles, practically forms a complete ring or halo around the nucleus (fig. 5).

The appearance of the different cell organelles is shown in figure 3 *et seq.* The ground substance of the cytoplasm contains a large number of electron-dense granules. These vary in density and are most difficult to

demarcate clearly from surrounding portions of the ground substance. They are sometimes quite isolated, but usually lie grouped in small, irregular clusters. In exceptional cases they form regular groups of 4 or 5 granules of bow- or clover-leaf shape. It has not been possible to measure their size exactly. The diameter seems to be about 150 Å. These granules occur in about the same numbers in all parts of the cell cytoplasm, except the above-mentioned light zones near the nuclear membrane or in the periphery of the cell (figs. 4 to 7).

Within these zones, especially in the region of the nucleus, it is sometimes possible to discern numerous fine fibrils. In general these show a clear parallel arrangment and appear to lie in pairs. The distance between the pairs is about 130 to 150 Å. The fibrils themselves are only about 30 Å thick (figs. 6 and 7). Most often they are concentrically arranged around the nucleus or around the center of the lighter zone near the periphery (figs. 5 to 7). It is only possible to trace them for a short distance in each section when they cease without joining or forming any bounded spaces. No clear border can be discerned between these lighter fibril-rich zones and other parts of the cell cytoplasm. These fibrils are difficult to observe and do not appear in all sections. In many cases they are only visible within a limited area, the remaining parts of the light zone do not show any definite ultrastructure and appear only as a fine-grained, homogeneous mass (fig. 4).

In the cytoplasm there is also a very large number of small, round or oval vesicles which are surrounded by distinct membranes of about the same dimensions as the cell membrane. The content of the vesicles is often lighter than the surrounding ground substance. They lie in small groups throughout the cytoplasm but they are most concentrated in the area nearest the nucleus, together with the structures that are considered to correspond with the Golgi apparatus. The so-called Golgi membranes appear as they do in other cells and therefore require no further description. These cell components are relatively small and there are only a few in comparison with certain other cells, *e.g.*, normal epithelial cells.

Further, the cytoplasm contains varying numbers of narrow, elongated "cisternae" of the type called "endoplasmic reticulum with rough surface" (*6*) or α-cytomembranes (*7*) [figs. 3 and 5]. These organelles are also relatively sparse but are remarkably long and narrow and have a large number of electron-dense granules on the outer surface. No definite relationship between these components and the mitochondria has been found.

The mitochondria are quite small, elongated, and sometimes hooked or branched. The inner membranes, the "cristae," are somewhat irregularly arranged but usually lie at right angles to the outer membranes. These "cristae mitochondria" are few and seldom stretch right across the whole of the mitochondrion. The inner substance is without structure and appears to have the same density as in other cells. In many mitochondria there may be seen one or more small, apparently empty vesicles surrounded by a thin membrane, probably remains of cristae (figs. 3 to 6). Electron-

dense bodies which are sometimes in the mitochondria of other cells, *e.g.*, the tubular epithelium of the kidney, have not been observed in these tumor cells.

The nucleus is bounded by a double membrane whose inner lamella seems to have so close a relationship with the electron-dense granules constituting the main part of the nucleus that it must be considered as the nuclear membrane proper. The outer lamella has the same dimensions as the outer membrane of the cell. Gaps or pores may be seen here and there in both lamellae. It is sometimes possible to see a fine thread or lamella running through the pores. The nucleus consists of a great number of electron-dense granules of approximately the same size and appearance as the granules in the cytoplasm. They are concentrated in the nucleoli and on the inner surface of the nuclear membrane.

The cell membrane is usually homogeneous, but it is sometimes possible to discern a lighter midzone between two dense peripheral zones as is found in other cells, Occasionally cleavage of the cell membrane into several fine fibrils can be seen. This is most likely due to an artifact caused by oblique cutting. Folds in the cell membrane with connections to the vesicles of the cytoplasm have not been observed.

DISCUSSION

These observations are in good agreement with those previously made by Selby *et al.* (*1*) and Epstein (*2*). We find the same organelles as in other cells and certain of these appear as they do in normal cells that have a greatly different function. Those structures generally considered to correspond with the Golgi apparatus (*8–10*) and the small vesicles included in the "endoplasmic reticulum" (*6*, and others) are examples of this. Other details in the structure of the cell cytoplasm, however, differ greatly from those seen in normal cells and deserve closer inspection.

Most important are the lighter areas close to the nucleus or in the periphery of the cytoplasm with their very fine fibrils. Similar structures were first described by Bessis and Breton-Gorius (*11*) in myeloblasts and promyelocytes of myelocytic leukemia. These authors remarked on the typical half-moon or ring formation of the areas, the concentrically arranged fibrils, and the absence of other cell components in these regions. They asserted that these zones are thus quite distinct from the nucleus and the Golgi zone. The authors considered that similar changes occurred in virus-infected cells and wondered whether these structures were caused by such an infection, even if evidence could not be shown.

Similar observations have been made by other authors (*12*). Rouiller *et al.* (*13*) showed the occurrence of the same structures in an experimentally induced endothelioma, Wessel and Bernhard (*14*) in a Yoshida ascites tumor, and Bernhard and de Harven (*15*) in a Rous sarcoma. Epstein (*2*) found that the fibrils in a Sarcoma 37 were less regularly arranged and that they were localized to the "centriosome region," *i.e.*, the region around

the centrioles. We could not find any structures that could be interpreted as centrioles in the vicinity of these fibrils nor does their partly concentric arrangement around the nucleus fully agree with Epstein's (*2*) assumption. Porter (*16*) and Gross *et al.* (*17*) observed similar, although probably coarser fibrils, which they interpreted as portions of mitotic spindles. According to Porter (*16*) these latter structures may sometimes be double, and Gross (*17*) asserts that they consist of two darker, outer lamellae with a lighter zone between them. Fine fibrils near the nuclei in ganglion cells were also observed by Palay and Palade (*18*). They state that the fibrils correspond to the so-called neurofilaments seen under the light microscope. It is remarkable that these fibrils in the cell cytoplasm have been seen only in tumor cells and in cells of mesenchymal origin. Nor do they occur in all cells of the investigated material [3–30% according to Bessis and Breton-Gorius (*11*)]. It seems unlikely that they are artifacts, as Epstein presumes, since they appear so regularly. One possibility is that they only occur in a preparatory stage of prophase to form mitotic spindles and are thus concerned with nuclear cleavage. We have, however, been unable to find any signs of initial mitotic cleavage in the nuclei of cells containing these structures.

In a recent paper by Edwards *et al.* (*19*) similar fibrils were described in cells from a human bronchiogenic carcinoma cultivated in the cheek-pouch of hamsters. The diameter was 90 Å and their size and number increased with aging and necrobiotic changes of the cells.

The proportion of small electron-dense granules in the cytoplasm is very high. As has been previously asserted by Palade (*19*), among others, the occurrence of such granules is connected with characteristic basophilic staining a high content of ribonuclear protein due to qualities which are typical for the cells of MC1M tumors. The so-called α-cytomembranes are rarely found in the cytoplasm, which supports Palade's assertion (*19*) that the basophilic character of the cells has nothing to do with these membranes, as such, but with the granules attached to their surfaces.

Viruslike particles such as those observed in ascites tumor cells by Selby *et al.* (*1*) were not observed in our material.

The appearance of mitochondria is naturally of much interest in cells with such active growth and unusual metabolism as ascites tumor cells. In general they have the same structure as other cells with a double outer wall and transverse cristae. They vary but little in their appearance. Particular attention should be given the frequent occurrence of light areas which seem to be situated within the cristae. Probably these areas contained a very soluble substance which disappeared during preparation. Their function is unknown. In the mitochondria that contain light areas the other parts of the organelle have a normal appearance. Inflated or swollen and empty mitochondria are not found in the ascites cells, as opposed to solid tumors obtained by subcutaneously transplanting ascites cells. This phenomenon will be discussed in a later paper.

The occurrence of many so-called lipide granules is noteworthy. The extent to which this may be connected with the life of the cells in an en-

vironment poor in oxygen and the results of such an environment on their metabolism are unknown.

The high concentration of electron-dense granules (nucleoproteins?) and the large nucleoli speak for an intensive metabolism and growth in the cells, facts which are well known. No special nuclear structures characteristic of these ascites tumor cells have, however, been observed. The occurrence of pores in the nuclear membrane was not as frequent in our material as in reports of Selby *et al.* (*1*) and Epstein (*2*). It is not yet known to what extent these pores have any connection or importance in exchange between the nucleus and the cytoplasm. We are thus unable to reach any conclusions from the varying occurrence of such pores. The appearance of the nucleus during mitosis has not been studied in this work.

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Plate 60

Figure 1.—General view of a tumor cell with a very large nucleus and 2 nucleoli, 1 lying close to the cell membrane. Numerous protrusions of protoplasm from the cell. Scattered mitochondria (M) in the cytoplasm in which there is a fairly large vesicle (V), *see* text.

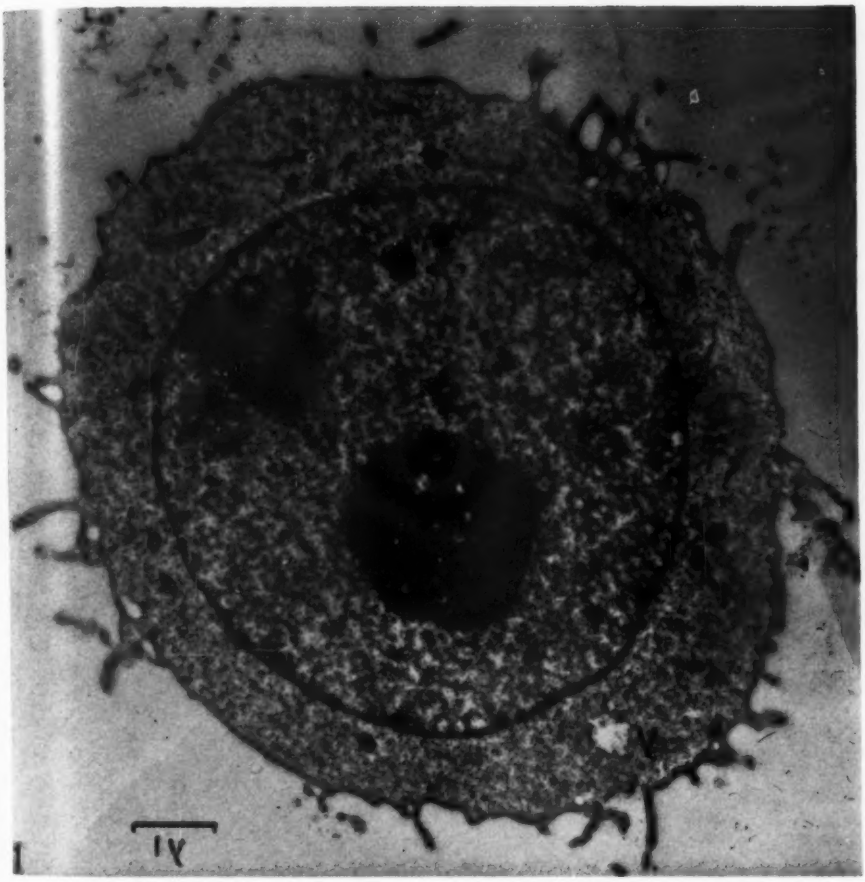

IV

PLATE 61

FIGURE 2.—General view of a tumor cell. Section shows only a small portion of the nucleus. Numerous mitochondria (M) and lipide granules (L) in the cytoplasm. *At right* cell shows a fairly large, ring-shaped, lighter area with almost no cell organelles. *Center* of this area contains a few mitochondria and lipide granules.

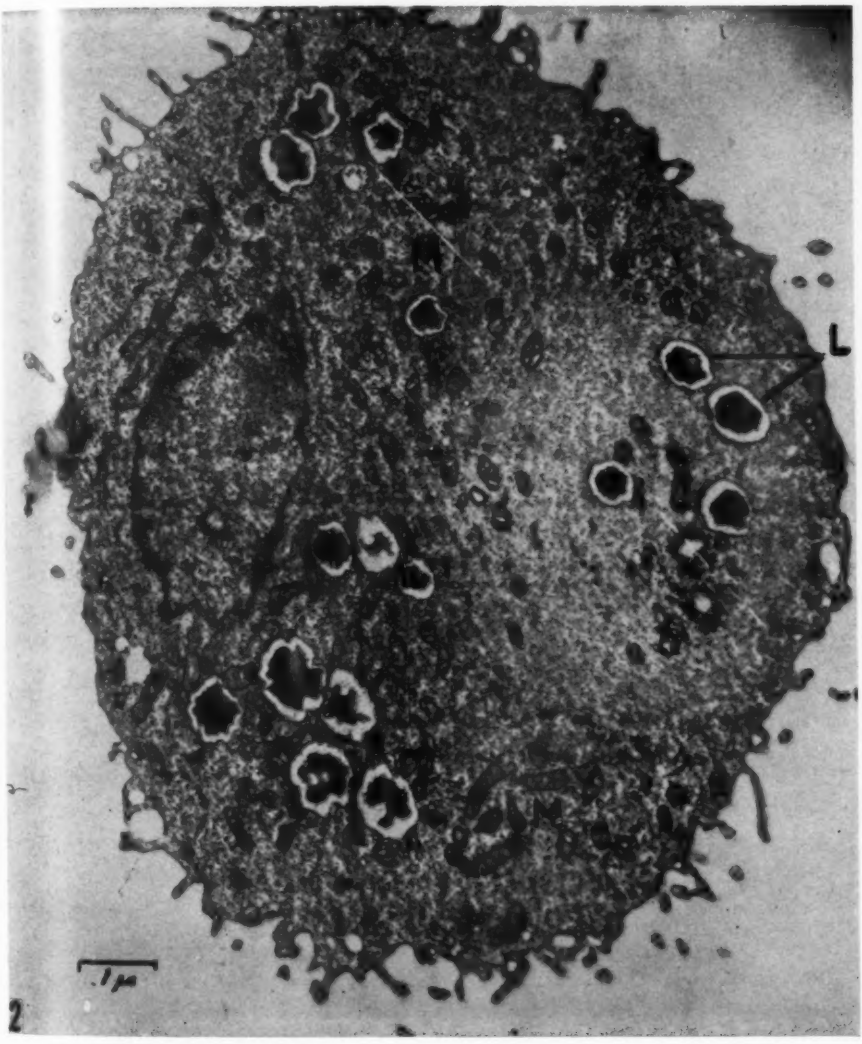

L
1μ
2

PLATE 62

FIGURE 3.—Detail of the cytoplasm with numerous small vesicles and lamellar systems which are believed to represent the Golgi apparatus (G). The nucleus (N) consists of 2 rather small parts, probably due to tangential sectioning. In the mitochondria many light areas are seen.

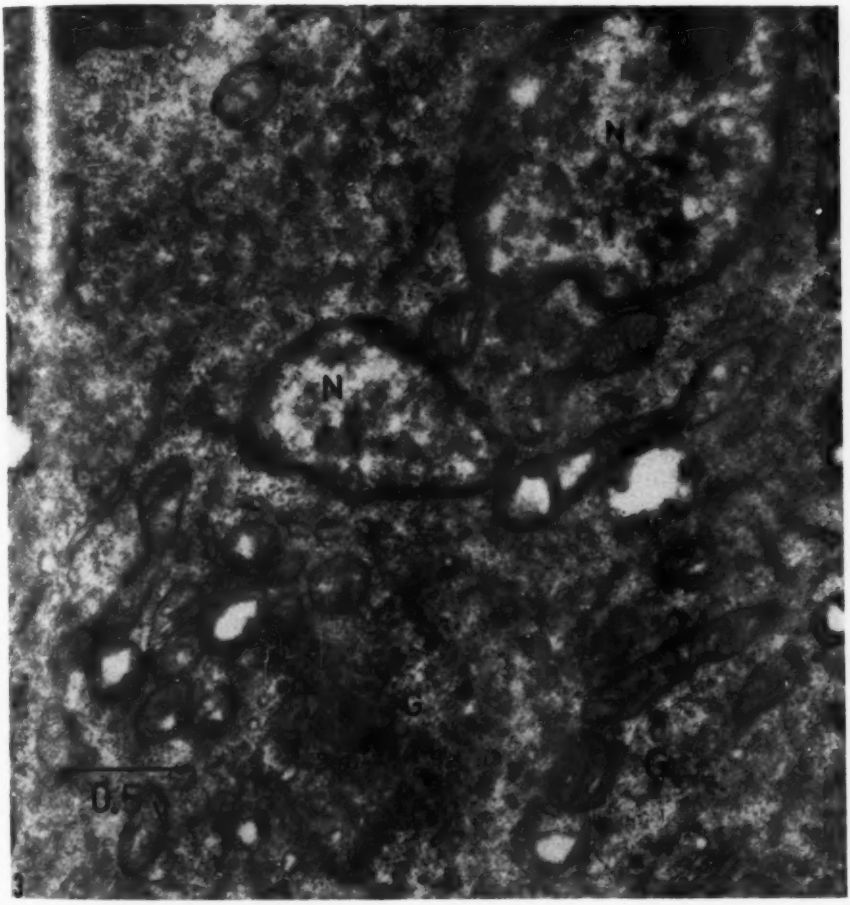

N
N
G
G
D.F.V.

PLATE 63

FIGURE 4.—Higher magnification of a light area corresponding to that seen in figure 2. No lamellar structure visible in this zone.

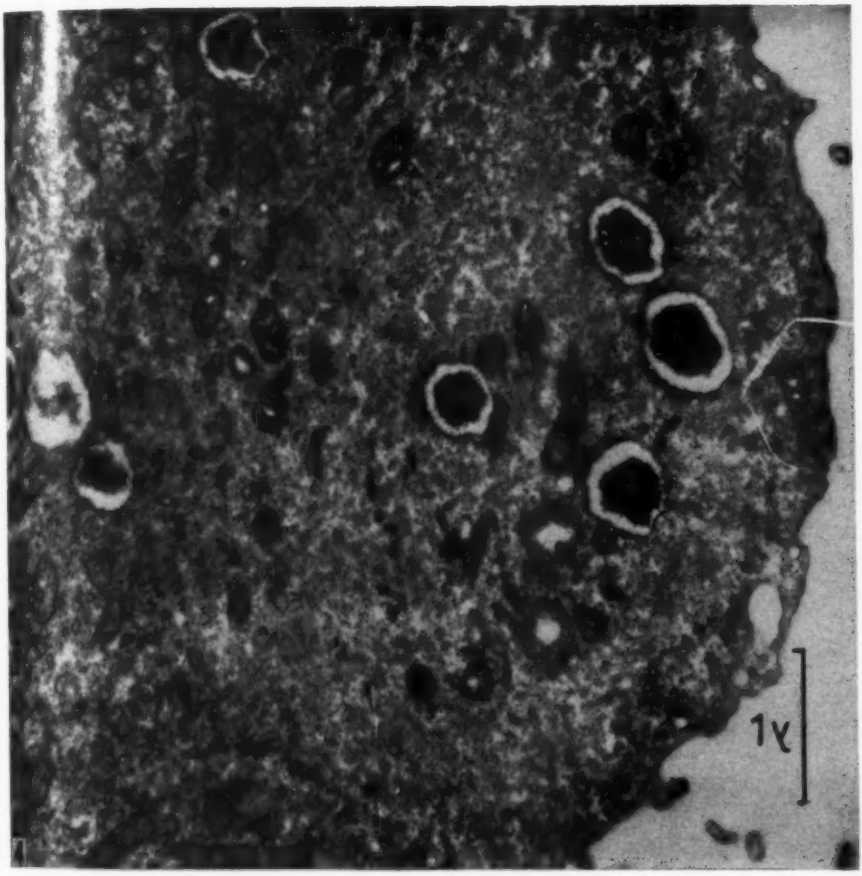

1γ

PLATE 64

FIGURE 5.—Part of a tumor cell in which the lighter zone (Z) is ring-shaped, and encircles the nucleus (N). Numerous mitochondria (M) and 1 or 2 α-cytomembranes (A) in the cytoplasm. In the mitochondria there are many lighter areas.

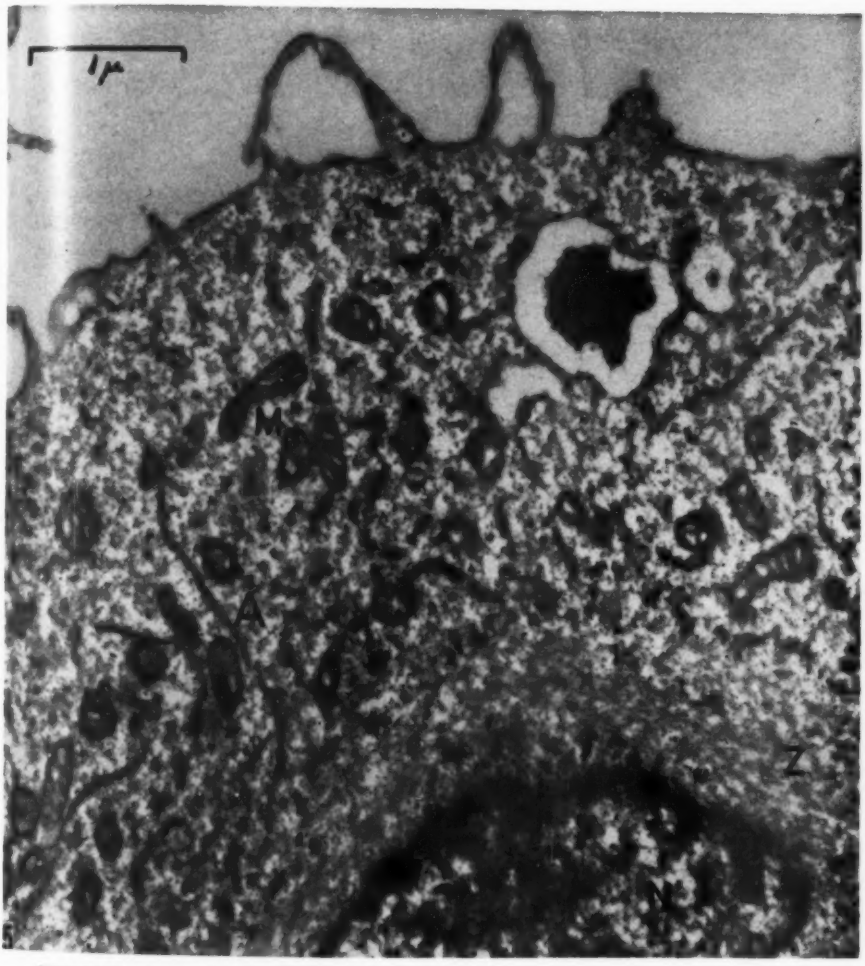

1μ
M
A
Z

PLATE 65

FIGURE 6.—Detail of the same cell as shown in figure 5. Close to the nucleus (N) the concentrically arranged, fine parallel lamellae in the light zone and further away in the cytoplasm several mitochondria (M). The lighter areas in the mitochondria are bounded by fine membranes which are probably remains of cristae mitochondria.

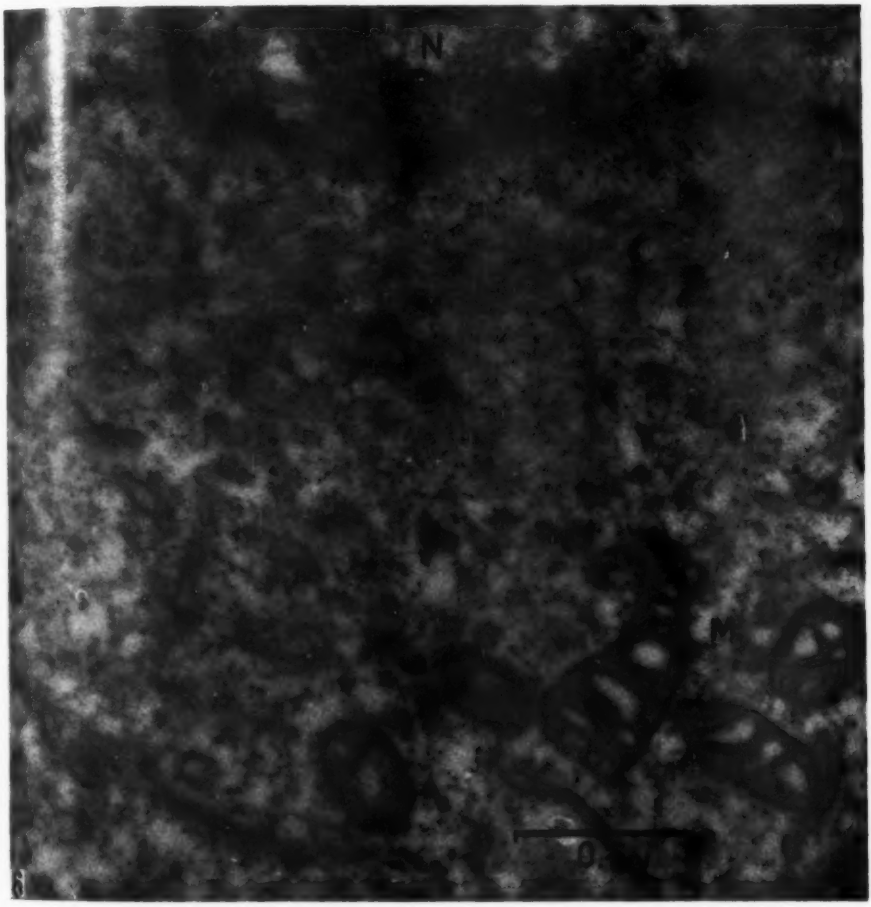
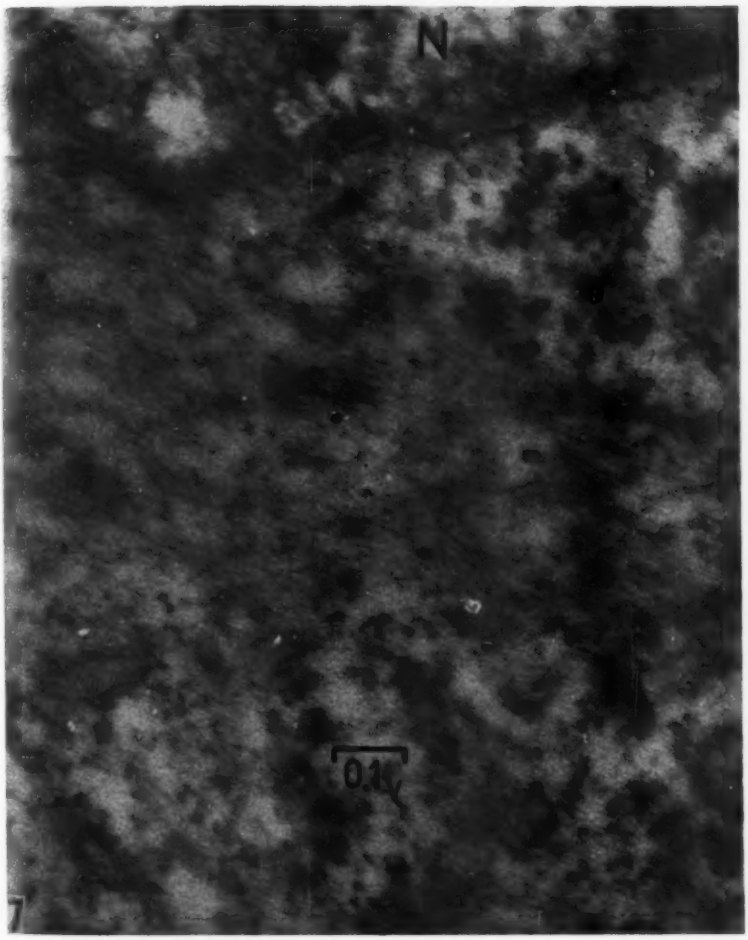

N

PLATE 66

FIGURE 7.—Detail of the light perinuclear zone with clearly visible, fine lamellae arranged in pairs. Spaces between pairs range from 130 to 150 Å and they are about 30 Å thick. Surrounding them are ribonuclear protein granules, which are numerous very close to the nucleus (N) and outside the light zone but sparse within it; they are never in direct contact with the lamellae.


N
0.1

Electron Microscopic Examination of the MC1M Tumor. II. A Comparison Between the Sublines M_{AA}, M_{AS1}, and M_{SS}[1]

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SUMMARY

Two variants of the MC1M tumor, M_{AS} and M_{SS}, growing in solid form in subcutaneous connective tissue of strain C3H mice were examined under the electron microscope. The M_{AS} tumor can be directly transferred to the ascites form, whereas the M_{SS} tumor can only be transplanted in the solid form. No morphological differences between the two variants were observed that could be correlated with the biological differences between them in regard to surface adhesion, cell organelles, or the construction of the cell membrane. Certain important differences were shown between MC1M tumor cells growing in the ascites and solid forms. The ascites cells are monomorphous and homogeneous, whereas the solid tumor cells are variable, mainly in the structure of the nucleus. The mitochondria are far larger and almost entirely devoid of homogeneous material in the solid-tumor cells. These differences are interpreted as a result of the differing milieux in which the tumors live.—J. Nat. Cancer Inst. 25: 523–545, 1960.

THE MC1M sarcoma was induced by means of methylcholanthrene in a mouse of inbred C3H strain. Three sublines were produced from this tumor by selective serial transplantation. The original solid subline (M_{SS}) was always obtained by transplanting the solid tumor subcutaneously. After intraperitoneal inoculation, the M_{SS} cells collect on the peritoneal surface, multiply, and infiltrate in different intra-abdominal tissues. There is little cell multiplication in the ascitic fluid formed by these peritoneal implants—the total number of free tumor cells in the fluid usually remains at about 10^4 to 10^5. By selective serial transplantation of the exudate intraperitoneally, another subline (M_{AA}) was produced from the same tumor in which the cells are adapted to grow mainly in the free, dissociated ascites form. Here the total number of cells in the fluid reaches 10^8 to 10^9, and the infiltrative, solid growth is much less pronounced. The M_{AA} cells give rise to solid tumors on subcutaneous inoculation; these are usually designated M_{ASn}, where n gives the number of passages in the solid form after reinoculation from the ascitic fluid. Rein-

[1] Received for publication February 2, 1960.

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oculated intraperitoneally, an M_{AS} tumor grows at once as a fully adapted ascites tumor, even after as many as 80 passages in solid form.

In a comparison between the properties of growth in these different types (*1*) it appeared that the M_{AS} and M_{SS} sublines also differed in their capacity to migrate to the lungs and infiltrate the subcutaneous connective tissue surrounding the periphery of the tumor. Furthermore, differences in the negative electric charges on the surface of the cells have been shown by Purdom *et al.* (*2*). The purpose of this investigation with the electron microscope is to discover whether there are differences between MC1M tumors growing in ascitic fluid (M_{AA}) and subcutaneously (M_{AS}), and also between ascites-adapted and unadapted subcutaneously growing tumors (M_{AS} and M_{SS}). In the first, it is a question of the same cell population growing in two different milieux, and in the second, of biologically different populations growing in the same milieu.

MATERIALS AND METHODS

The *ascites tumors* (M_{AA}) were the same as described in the first paper of this series.

The *solid subcutaneous tumors* consisted of 14 M_{AS1} transplants of the M_{AA} and 13 M_{SS} tumors derived by subcutaneous serial transplantation of the original methylcholanthrene-induced sarcoma. These transplants were examined when they had reached 2 to 5 mm. in diameter.

The animals were killed by fracturing the cervical vertebrae and parts of the subcutaneous tumors were extirpated as quickly as possible and placed in a large drop of cold 1 percent osmium solution, buffered to *p*H 7.2 The pieces of tissue in the drop of fluid were further divided into very small cubes and were then transferred to a small glass jar containing the same fixative. After fixing for 1, 2, or 4 hours, with several changes of the fixative, the material was washed for 1 hour in Tyrode's solution, dehydrated in 70 percent, 90 percent, 95 percent, and absolute alcohol and embedded in methacrylate (19 parts butyl and 1 part methyl methacrylate). Polymerizing occurred at 57° C. and continued for about 48 hours.

Four animals, 2 from each of the M_{AS} and M_{SS} groups, were anesthetized with 0.1 mg. of sodium pentobarbital (Nembutal) intraperitoneally. The subcutaneous tumors were exposed and covered with a large drop of cold osmium solution. Portions of these tumors were then extirpated in the osmium solution and immediately transferred to a glass jar containing the same solution. The procedure, as described, then followed. Thus in these cases fixation was begun *in situ* and the extirpation and division of the tumor tissue completed within 30 seconds. There was never an interval of longer than 3 minutes between killing the animal and completing the division of the tumor tissue into small cubes ready for the final fixation.

OBSERVATIONS

Figure 1 shows a general view of an M_{AS} tumor. The cells are clearly separated and have very little contact. Between them are large spaces in the tissue, some quite empty and some containing large or small cell fragments that probably belong to tumor cells. In other portions of the same tumor are seen subcutaneous connective tissue, scattered bundles of collagen fibrils, and isolated inflammatory cells—leukocytes and lymphocytes or plasma cells. The appearance of the nuclei varies considerably. In some cells one or more nucleoli are clearly seen, whereas in others the electron-dense substance is more diffusely arranged in the nucleus.

Figure 2 shows a corresponding portion of an M_{SS} tumor with the same loose structure. A disintegrating tumor cell is in the center of the picture.

Figure 3 shows a portion of an M_{AS} tumor with a more solid structure. Most of the cells are closely packed and in many places there are between them just fine fissures. The individual cells are easily recognizable, and only occasionally are they bound together by intertwined cell protrusions as is often seen in various normal tissues. Where 3 or more cells touch each other there are somewhat larger tissue spaces, and in these, small protrusions from the cell walls are visible. The electron-dense substance in the nuclei is plentiful, and diffusely arranged or gathered into irregular groups. Only one nucleus contains a distinct nucleolus. Mitochondria (M) are visible in a number of cells. There are no disintegrating cells in this region.

Figure 4 is an equivalent region in an M_{SS} tumor. The arrangement of the tumor cells is about the same as in figure 3.

Figure 5 shows the detail of a junction of 2 cells from a solid portion of an M_{AS} tumor. The cell membranes lie closely parallel for a distance of about 200 to 300 Å. In the cell cytoplasm there are many ribonuclear-protein granules in groups shaped like clover leaves. Several large mitochondria are visible. These are swollen and their contents are nearly homogeneous and are almost completely devoid of "cristae mitochondria."

Figure 6 shows a corresponding region in an M_{SS} tumor. It is impossible to follow the cell membranes completely in this picture. The arrangement of the cells, however, is exactly the same as in figure 5. The nuclei (N) are very large and contain many electron-dense granules which are especially concentrated round the nuclear membrane. The cytoplasm contains round or oval vesicles that have a diameter of 300 to 500 Å (endoplasmic reticulum).

Figure 7 shows 2 cells in an M_{AS} tumor that are more loosely connected. The widening of the intercellular space (U) occurs much more frequently and is larger than in figures 5 and 6. Certain cell organelles are visible in the cytoplasm and look about the same as those in the previous figure.

Figure 8 shows another somewhat looser junction between the cells in an M_{SS} tumor. In the upper-left corner the cell relationship corresponds to that seen in figure 7, whereas in the center there are many cell pro-

trusions intertwined, to a certain extent, in the intercellular spaces. The spaces show protein precipitated from the interstitial fluid.

Figure 9 illustrates the extensive separation of cells in an M_{AS} tumor. Here there is hardly any contact between the cell surfaces. In the cytoplasm there are electron-dense ribonuclear-protein granules, which here show a very distinct grouping in small irregular clusters. The mitochondria (M) are large and "empty" as in figure 5.

Figure 10 shows a corresponding view in an M_{SS} tumor with cells which are almost completely free. Thick protrusions from the cell membrane extend into the intercellular space.

Figure 11 further illustrates the different cell organelles in an M_{AS} tumor. The nucleus occupies the greater portion of the visible part of the cell. It is bounded by a double membrane of the same dimensions and appearance as in most other types of cell. The different cell organelles appear very clearly, especially the large, electron-dense lipide granules whose contents are neither precipitated nor particularly shrunken. The various small vacuoles and membranes in the cytoplasm are likewise very distinct. Here it is justifiable to assume that there has been a minimum of disturbance during preparation. In spite of this the mitochondria are very large and nearly empty, with the same appearance as in figure 5.

Figure 12 shows some well-preserved lipide granules in a comparatively thick section of an M_{SS} tumor. Because of its thickness, however, the remaining cell organelles are not distinct.

Figures 13 and 14 illustrate the appearances of the different cell components in M_{AS} and M_{SS} tumors. The empty appearance of the mitochondria and the agglomeration of the ribonuclear-protein particles in small clusters are clearly seen. Figure 13 also shows the cell membrane. It is single-layered and has a thickness of about 50 to 75 Å. It appears to be split into several lamellae, but this is probably caused by oblique sectioning.

DISCUSSION

Cell density and surface adhesion.—Earlier investigations with the light microscope have shown that the M_{AS} tumors, especially at the periphery, have a loose structure with considerably less surface adhesion between cells than the M_{SS} tumors which grow more compactly (*1*).

Electron microscopic investigations by Dalton (*3*) have shown that subcutaneous tumors (Sarcoma 37) compared with the same tumor in ascites form have few or no cell protrusions.

Purdom *et al.* (*2*) have studied the speed of migration of MC1M tumor cells in electric fields. They found that cells from a tumor which had lived in ascites form for many generations (M_{AA}) migrate considerably faster, *i.e.*, have a considerably greater negative charge than isolated cells from the solid M_{AS} tumor. These, in turn, have a greater negative charge than cells from the M_{SS} tumor. M_{AS} cells which have been retransplanted intraperitoneally recover, after only a few generations, the same high

charge found in cells from a tumor maintained in the ascites form for a long time. They conclude that the ability to convert to ascites form, like the tendency to spread by migration, is correlated with the strength of the negative charge.

It might be presumed that this difference in negative charge is matched by structural differences, *e.g.*, in the cell membrane, which cause varying surface adhesion between cells. This would explain why the M_{AS} but not the M_{SS} tumor can immediately be converted to the ascites form.

We have tried to determine whether such structural differences between the two types of tumors exist, but no such differences have been observed in the cell membranes. A single membrane, about 70 Å thick was seen in both types.

Also, we have been unable to show any reliable difference between the 2 tumor forms as to cellular density or connection between the cells (surface adhesion). There is of course no yardstick one could use making such a statement. Our conclusion is based solely on the subjective appraisal of a large number of sections from different portions of the tumors in each group. We have endeavored, as far as possible, to include sections from both the center and the periphery of all tumors. We have observed important differences in cell density between various tumors independent of tumor form and considerable variation in different areas of the same tumor. There are areas with few cells having an extremely loose structure in which the individual cells are very like ascites cells (figs. 1 and 2) and areas with the cells closely packed (figs. 3 and 4). The surface of contact between the cells may be either quite smooth or have numerous, branched protrusions of cytoplasm interwoven in a very complex pattern. Intermediary forms are found between these extremes (figs. 5 to 10).

We consider that the particularly loose cell structure observed in the center of most of these tumors is a sequel to an initial cell disintegration that is already in process a few days after transplantation and that increases with the age of the tumor. Surrounding this necrobiotic zone there is a more compact structure containing tightly packed, living cells. In young tumors this area covers nearly the whole tumor. Finally, in the most peripheral portions there is again a looser structure, with large or small spaces between the cells, in which the remains of disintegrating connective tissue or subcutaneous musculature are seen—the infiltration zone.

As stated, we have been unable to show any reliable differences between M_{AS} and M_{SS} tumors in regard to the size, distribution, or general appearance of these zones.

A comparison between the MC1M tumor in ascites form M_{AA}, described in a previous paper, and the same tumor in the solid form, M_{AS} or M_{SS}, does, however, show certain important differences. The ascites tumor cells are highly monomorphic. This is true of the cells as a whole, as well as of individual cell organelles to an extent that is seldom seen in solid tissue. The solid-tumor cells have greater variation, as is usually true of malig-

nant tumor cells. Large numbers of cells in various stages of disintegration are seen chiefly in central portions, and the variations in form, size, or content of electron-dense substance are also much more marked than in the ascites cells.

Cell organelles.—In general, the organelles are not very different. Large, lipide granules, as well as the different structures included in what has been called the endoplasmic reticulum by Palade (*4*), and others, are present to the same extent and have the same appearance in both the ascites and solid-tumor cells. There seem to be no reliable differences between the M_{AS} and M_{SS} tumors in this respect either.

We have not been able to find any clear divergencies among M_{AA}, M_{AS}, and M_{SS} tumors in the occurrence of ribonuclear-protein granules in the cytoplasm.

Mitochondria.—In solid-tumor cells the mitochondria are much larger than those in ascites cells and are almost empty (fig. 3 *et seq.*). These changes were observed in sections from the center and the periphery of the tumors. Rhodin (*5*) and Zetterqvist (*6*) have described similar changes in the mitochondria of different epithelial cells and consider that they are due to postmortem change through incomplete or delayed fixation. In none of our cases, however, was the time between the killing of the animal and the final cutting of the tissue and commencement of fixation longer than 3 minutes. There is little likelihood that such marked postmortem changes in the mitochondria could arise in such short time. To achieve as rapid and complete fixation as possible, the fixation in 4 animals was started in the living animal, as described. Even in these tumors the mitochondria looked the same. Furthermore, electron micrographs show that all other cell organelles, including the lipide granules are very well preserved. It therefore seems unlikely that only the mitochondria were altered during preparation. Similar mitochondrial structures have been seen in different types of tumor cells by several authors (*7–11*).

Gansler and Rouiller (*12*) showed that liver-cell mitochondria appear empty when starved and that these changes are reversible. These observations were confirmed later by Bernhard and Rouiller (*13*) and others. We are therefore convinced that the appearance of mitochondria in solid tumors is not due to incomplete or careless fixation but to the functional state of the cells. Variations do occur since the content of mitochondria of certain cells is somewhat better-preserved. We have been unable, however, to find any clear, unambiguous difference between the mitochondria of the M_{AS} and M_{SS} tumors.

The mitochondria of the ascites cells, M_{AA}, on the other hand, were markedly different. As described in the previous paper, the M_{AA} mitochondria are rather small, with well-preserved contents and regular, transverse cristae mitochondria. Small, light areas between the separated membranes of the cristae were seen in many mitochondria. Here there was probably some easily soluble substance which was dissolved away during preparation.

A purely morphological investigation naturally cannot determine the importance of this difference between the mitochondria of ascites and solid tumors. We do know that the mitochondria are the source of a large part of the enzyme production of the cell. It may also be presumed that cells living in the ascitic fluid have a different metabolism and enzyme production than those living in a more natural milieu. Thus it is very possible that this difference in the appearance of the mitochondria is dependent on the different milieux in which the cells exist. It is hardly possible that the difference in structure is constitutional, which makes some cells able to live in ascitic fluid, others not. If this were true, there would be marked differences between the structures of the mitochondria in the M_{AS} and M_{SS} tumors.

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PLATES

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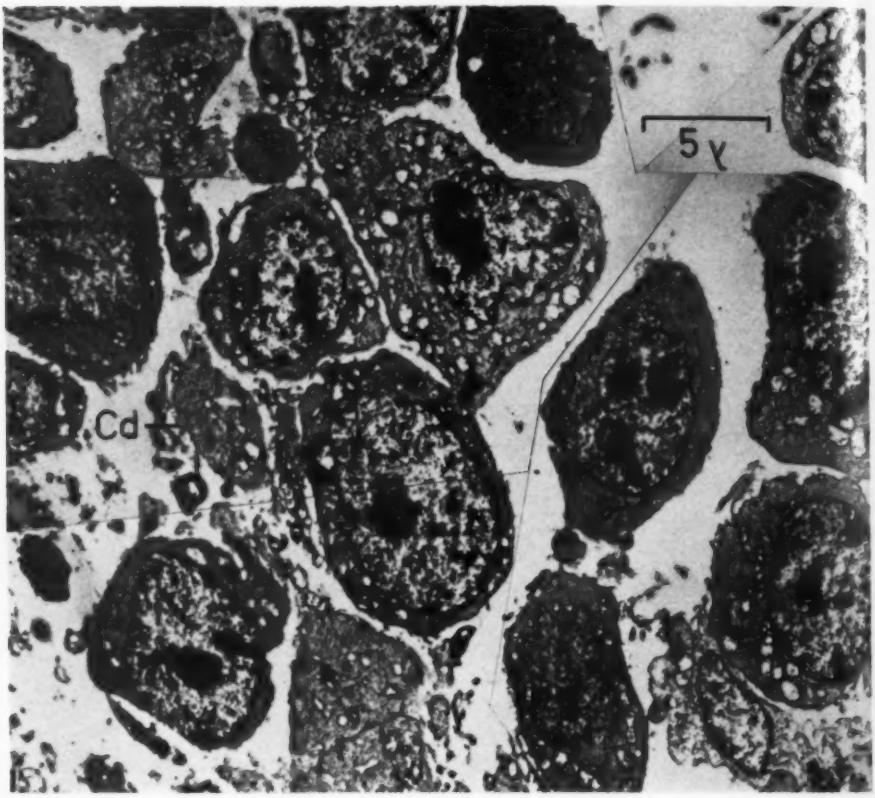

FIGURE 1.—General view of an M_{AS} tumor. Very loose structure, with individual cells lying almost free. Some visible cell fragments (Cd) probably are disintegrating tumor cells. Considerable variation in the appearance of the nuclei. No recognizable stroma cells.

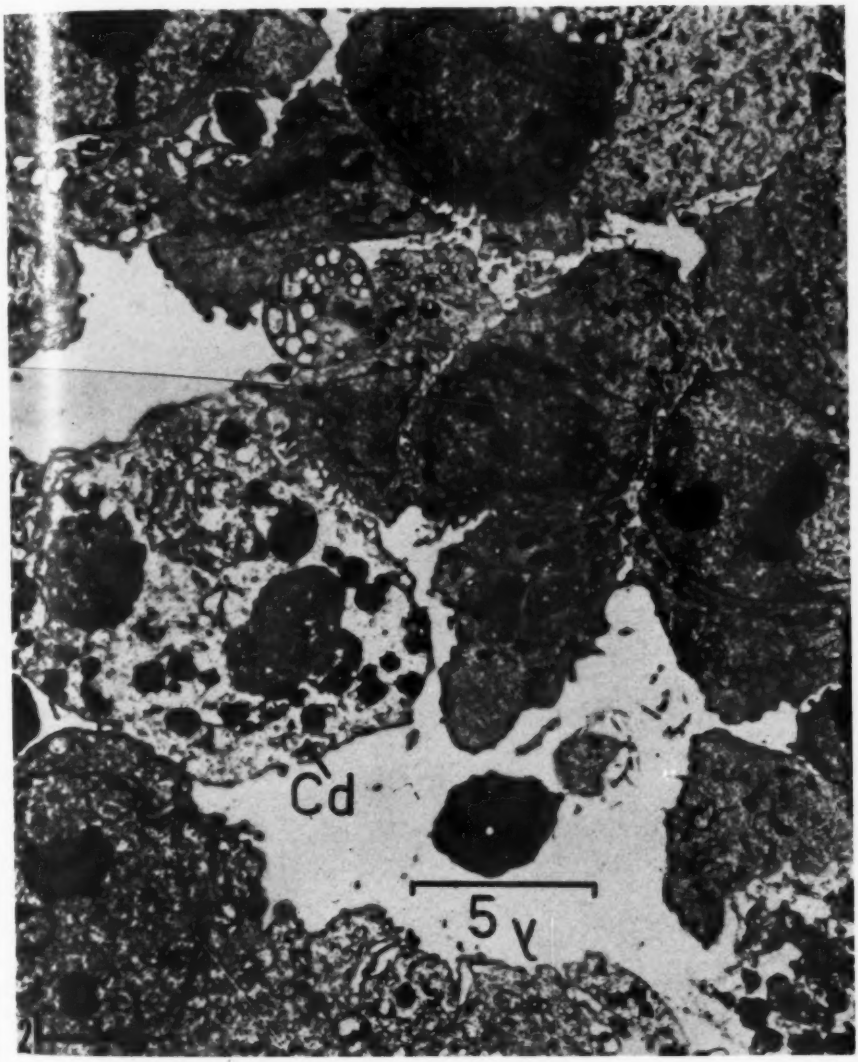

FIGURE 2.—Corresponding view of an M_{88} tumor. Single, disintegrating cell *left* (Cd). In some areas cells are loosely connected and in others they lie closely packed. Much variation in the appearance of the nuclei. Distinct nucleoli.

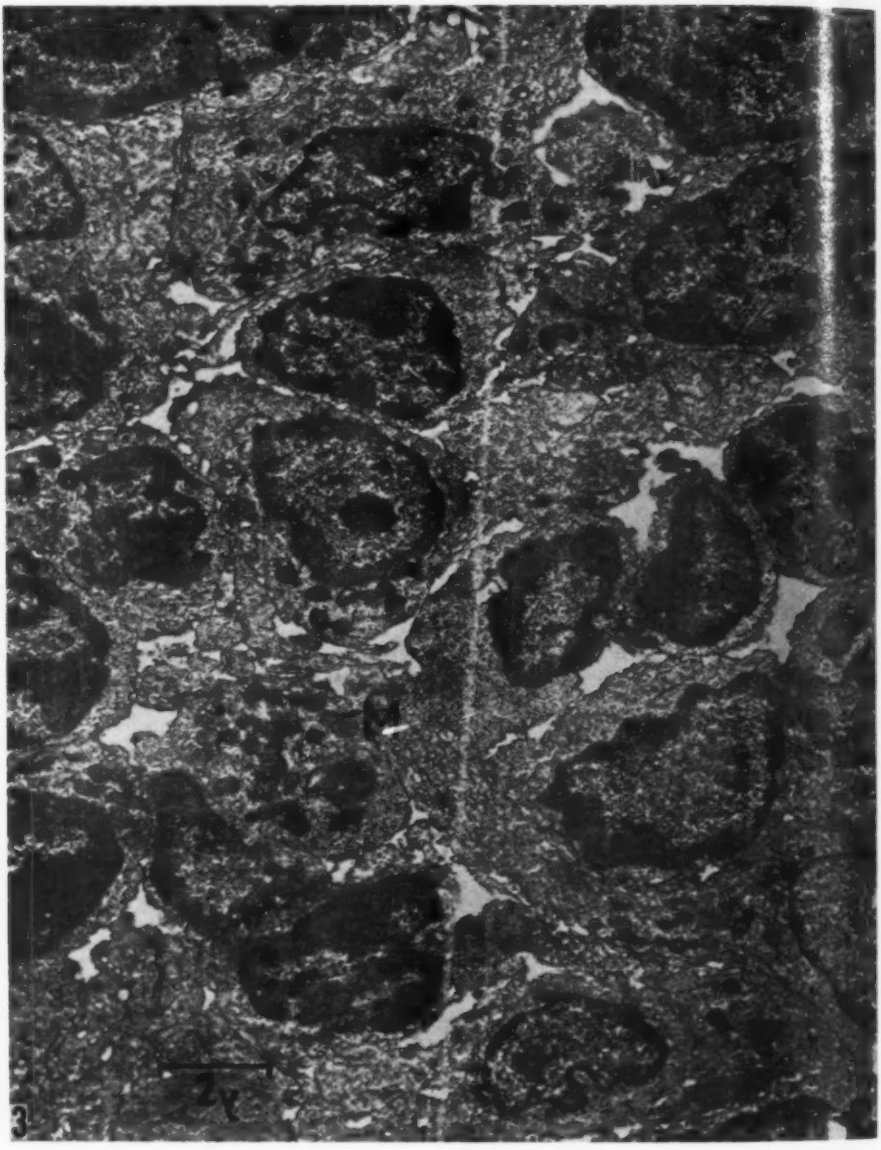

FIGURE 3.—Solid portion of an M_{AS} tumor. Differences in relationships between cells can be seen quite clearly. In general, however, this structure is considerably more closely packed than that in figure 1. Much variation in the appearance of the nuclei.

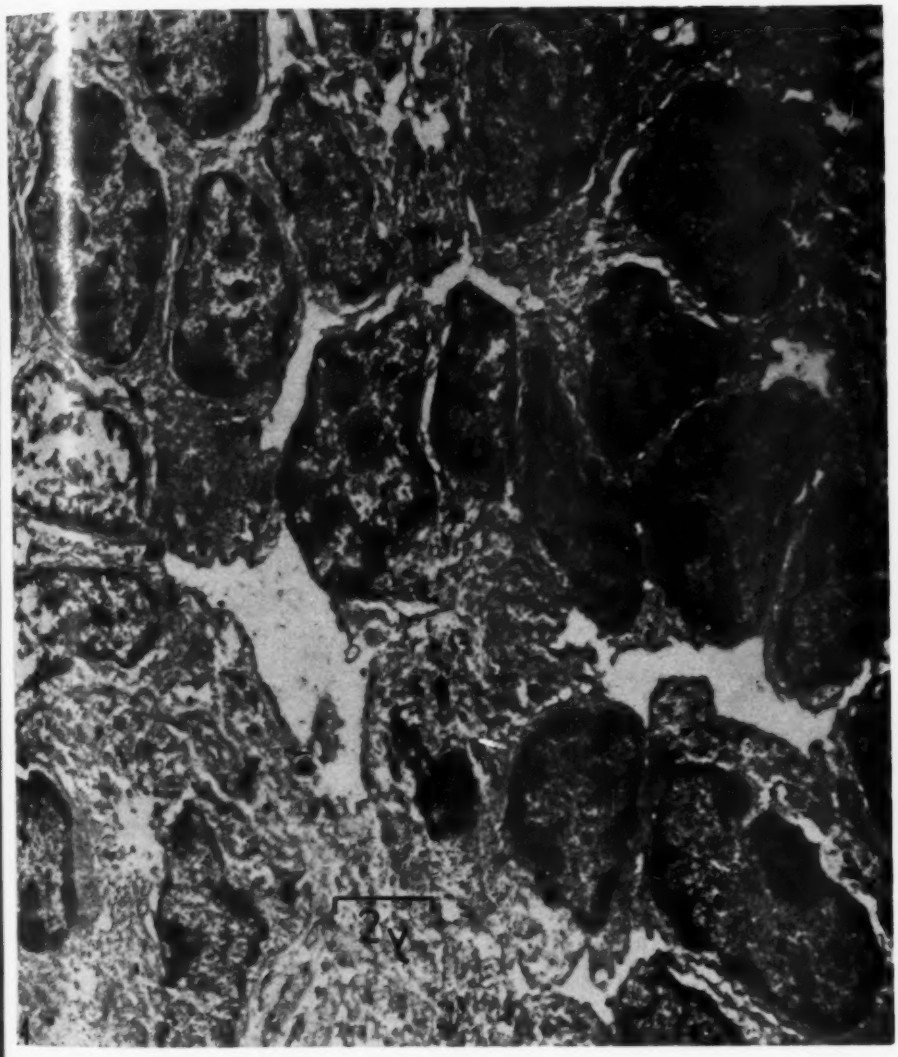

FIGURE 4.—Corresponding view of an M_{88} tumor.

FIGURE 5.—Detail of 2 cells from an M_{AS} tumor. The cell membranes lie closely parallel. The mitochondria (M) are nearly empty. Only scattered remains of "cristae" visible. Other cell organelles, such as ribonuclear-protein granules and small vesicles, are well preserved.

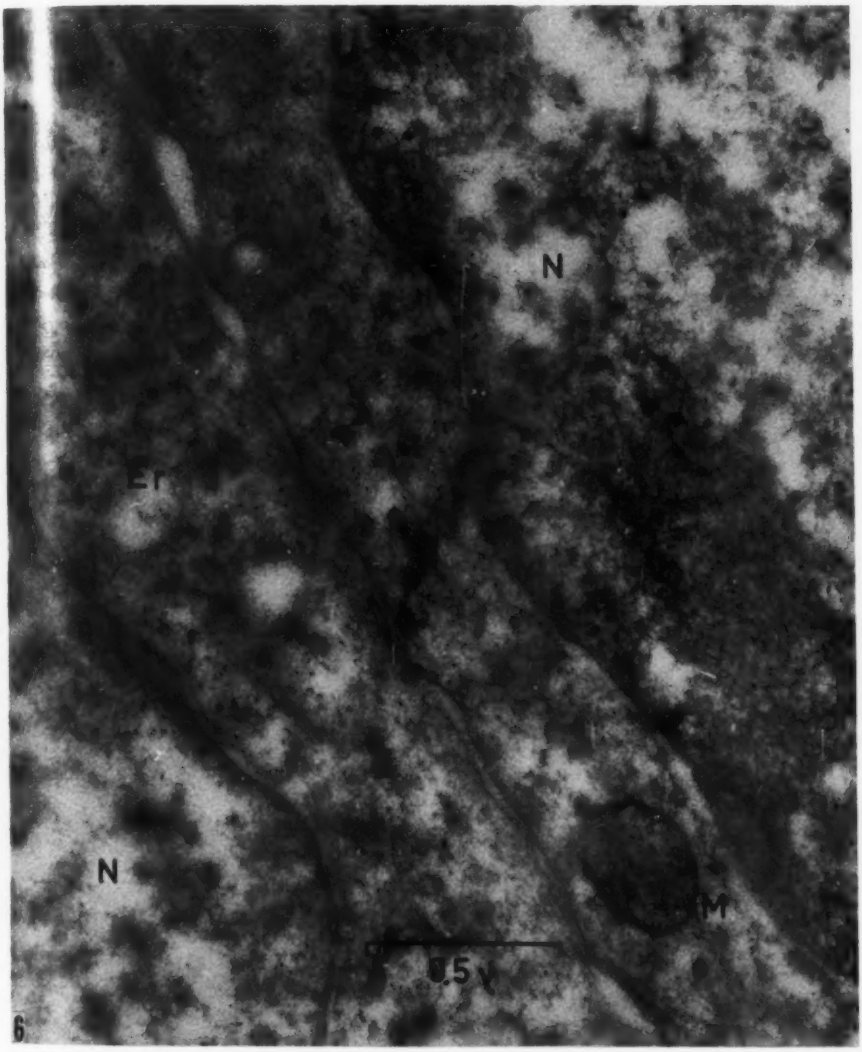

FIGURE 6.—Corresponding view of an M_{88} tumor. At those points where the cell wall has buckled inward the intercellular space is wider. Elsewhere the membranes are closely parallel. A single mitochondrion (M) has a slightly better-preserved structure than the one in figure 5. Also, several small vesicles belonging to the so-called endoplasmic reticulum (Er).

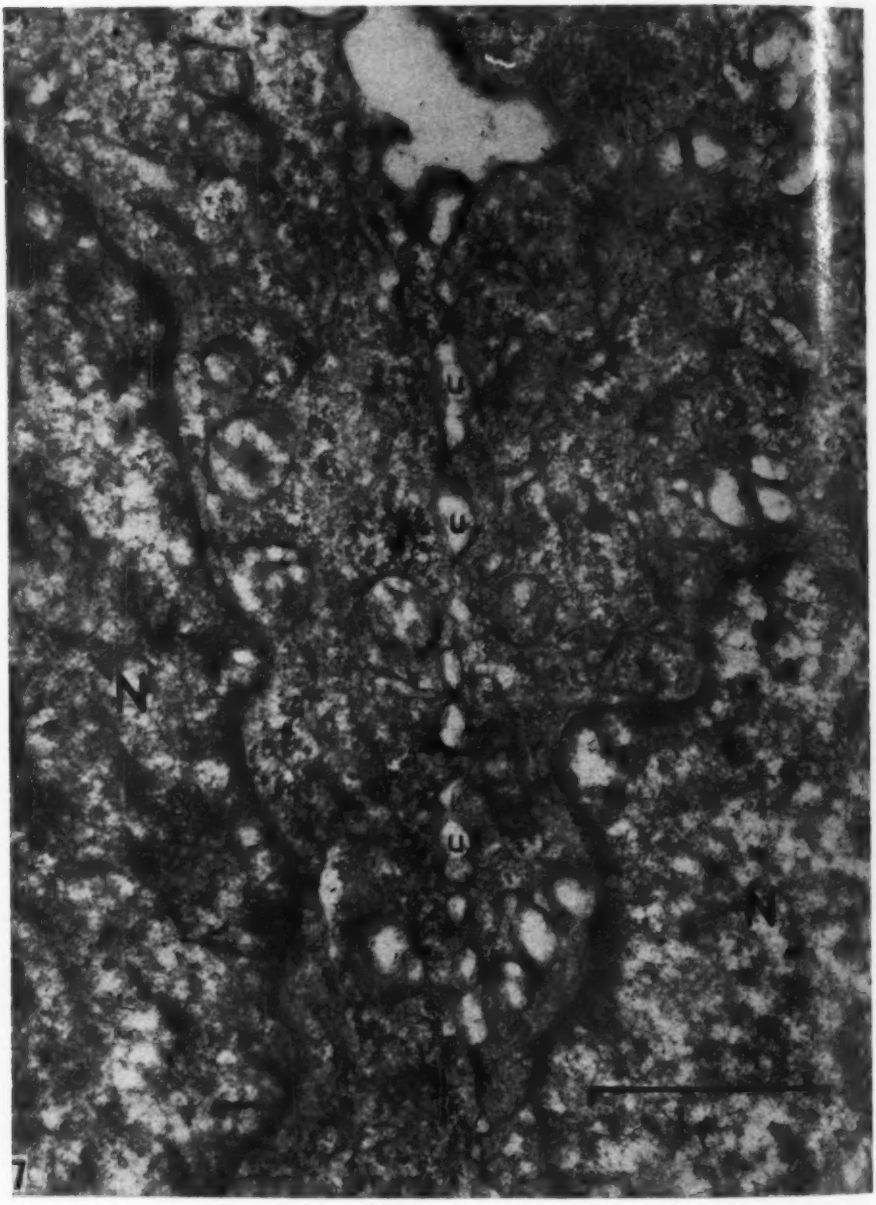

FIGURE 7.—Two cells from an M_{AS} tumor more closely connected than those shown in figures 5 and 6. The intercellular border in the midline of the picture is a series of small spaces (U), much like a string of pearls.

FIGURE 8.—Corresponding view of cells from an M_{88} tumor. Here the tumor cells are connected much more loosely. Numerous cytoplasmic protrusions from the cell to *left*.

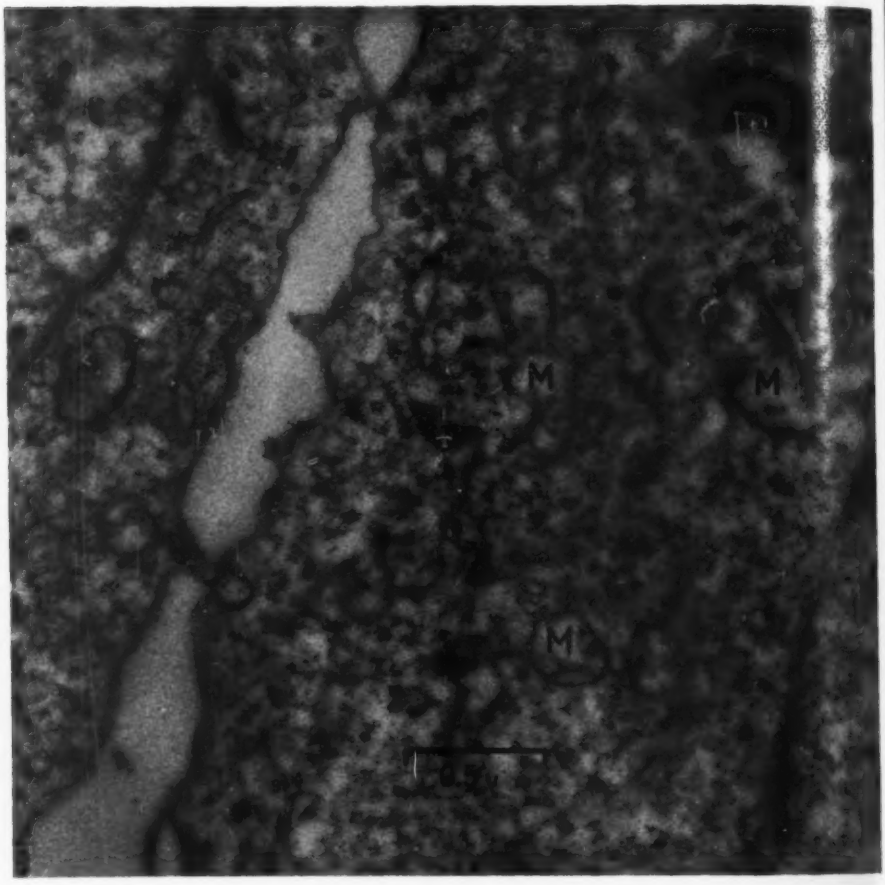

FIGURE 9.—Two cells from an M_{AS} tumor which have almost no connection. The cell organelles are well preserved and have prominent ribonuclear-protein granules. The mitochondria are nearly empty.

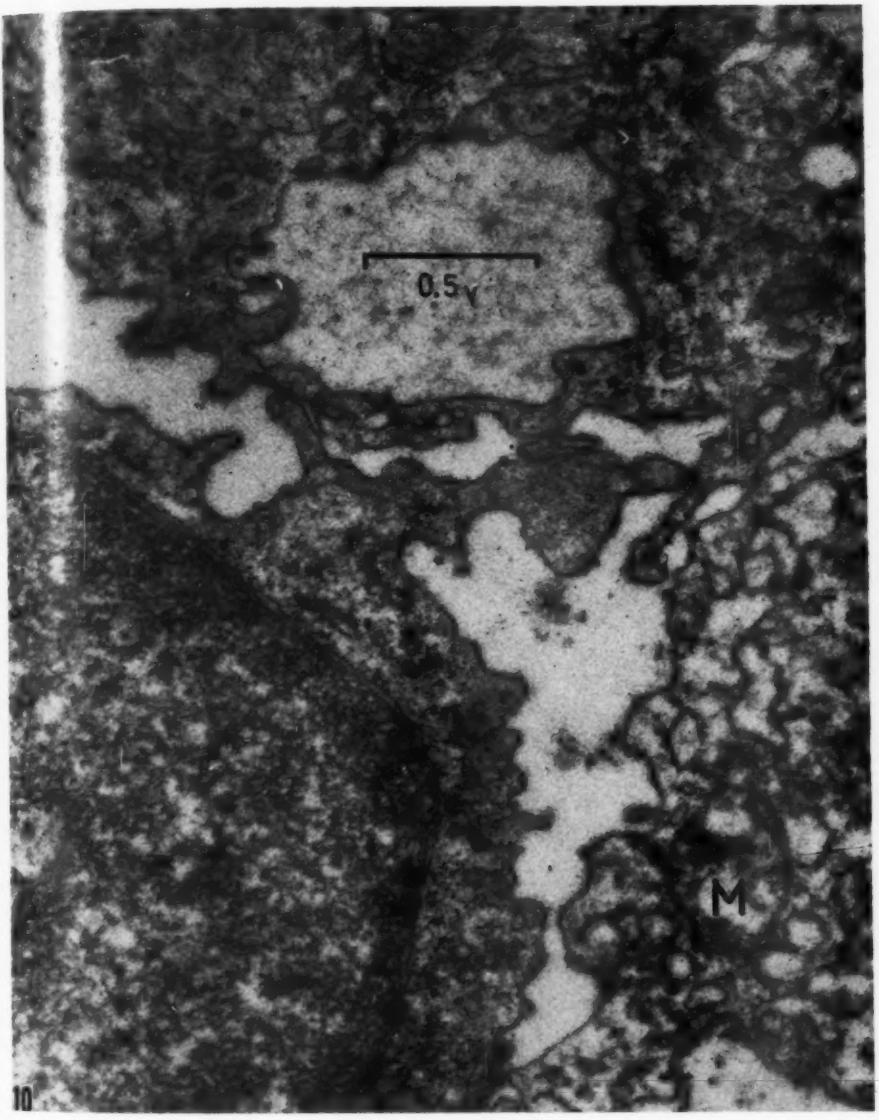

FIGURE 10.—Detail of the boundary between three cells of an M_{88} tumor. Several broad, branching, and intertwining protrusions from the cell surfaces. Otherwise the cells are separated by broad spaces. The structure of the nucleus is very well preserved but one mitochondrion (M) has changed.

FIGURE 11.—Two entirely separated cells from an M_{AS} tumor. Very similar to cells from a corresponding tumor in ascites form. The cell components, such as the large lipide granules, appear clearly, whereas all visible mitochondria (M) are vesicular.

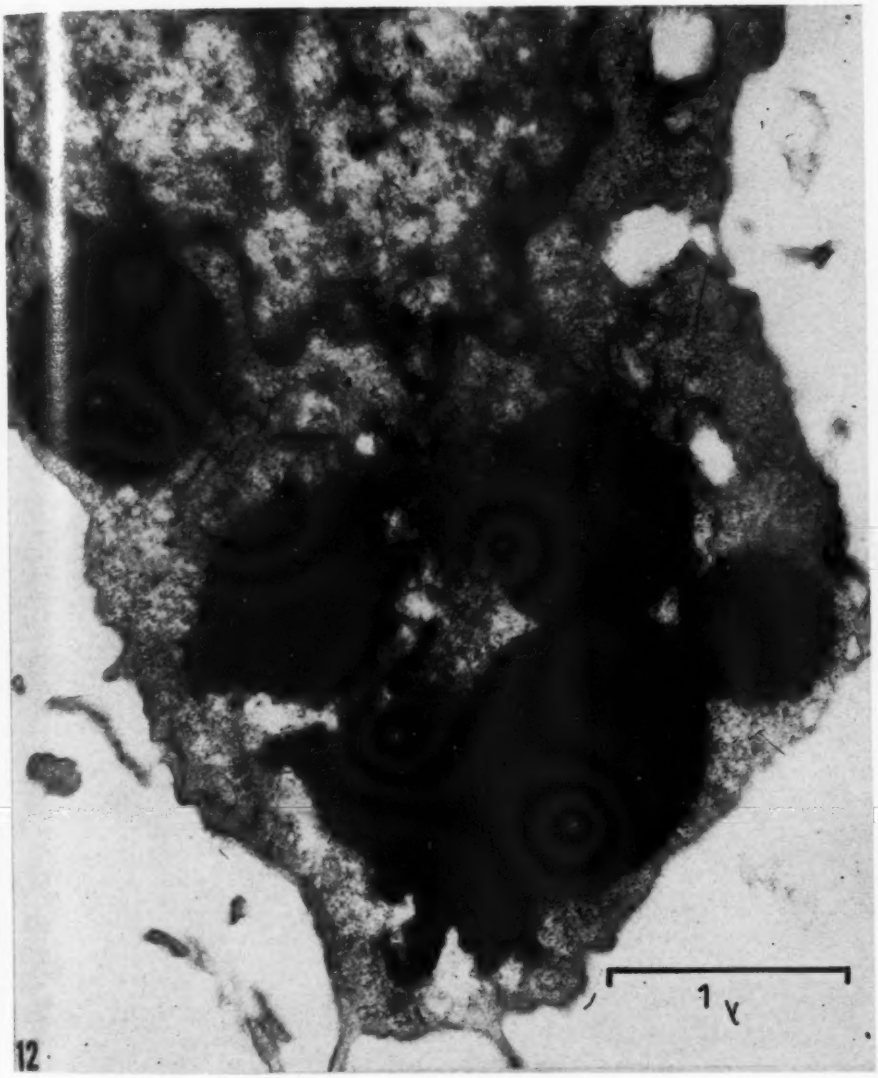

FIGURE 12.—Similar picture of a cell from an M_{ss} tumor with very prominent lipide granules (*see* text).

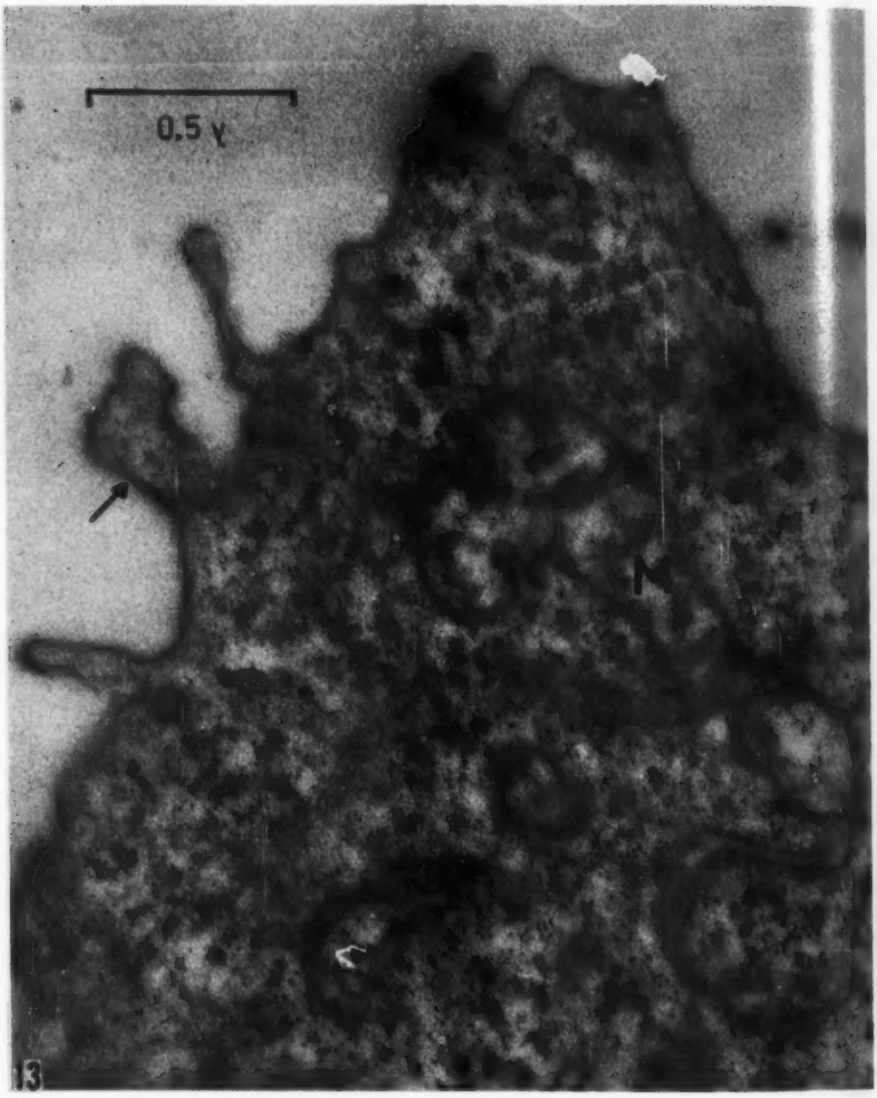

FIGURE 13.—Detail of a cell from an M_{AS} tumor. The cell membrane is clearly visible. The longitudinal cleavage (*arrow*) is presumably due to oblique sectioning. One large vesicular mitochondria with remains of "cristae."

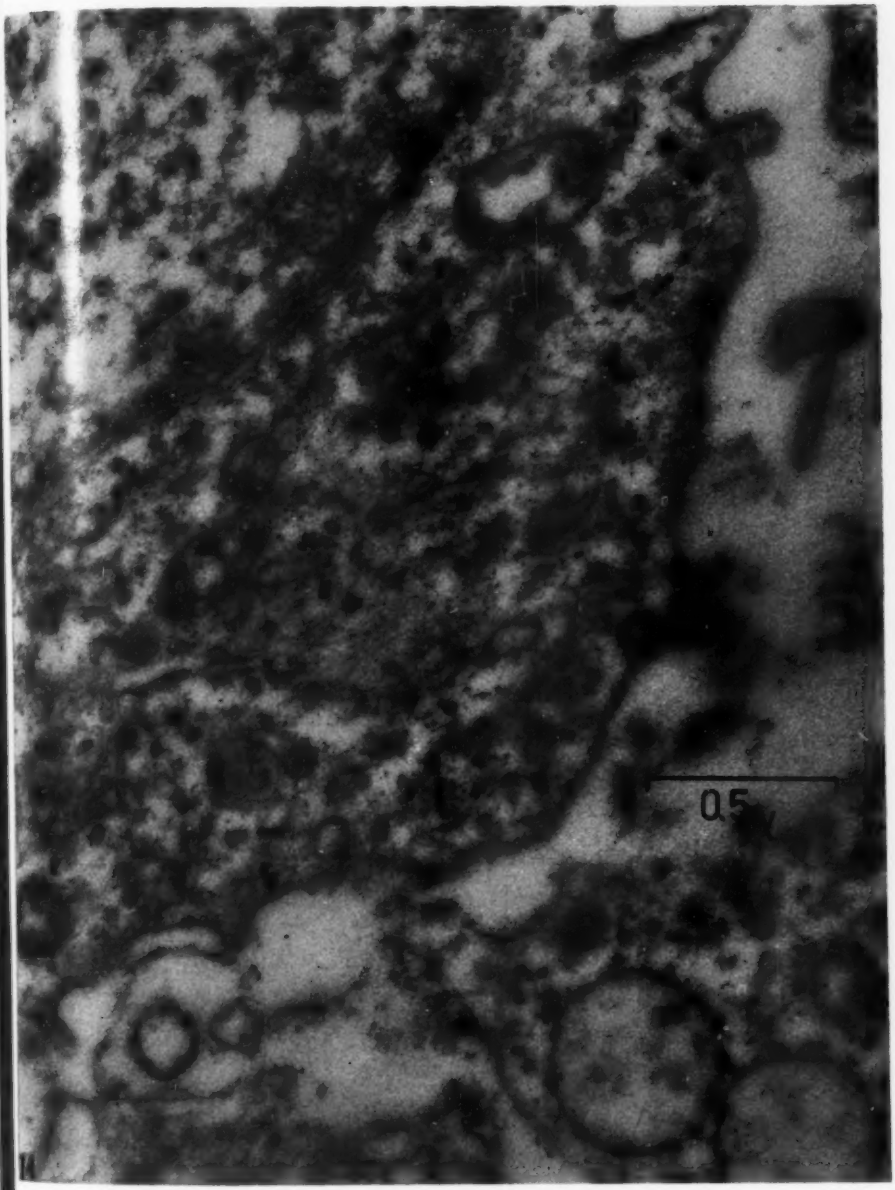

FIGURE 14.—Detail of a cell from an M_{88} tumor with cell organelles similar in appearance to those in figure 13.

An Ascites Tumor Derived From the Novikoff Hepatoma [1, 2]

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SUMMARY

A transplantable ascites tumor was derived for the first time from the Novikoff hepatoma. It has been carried as a free-cell population but can readily be converted to an island form.—J. Nat. Cancer Inst. 25: 547-555, 1960.

THE NOVIKOFF hepatoma, a solid transplantable tumor, originated in a Sprague-Dawley rat that had been fed a riboflavine-deficient diet containing *N,N*-dimethyl-*p*-phenylazoaniline (*2*). Since then it has been widely used in several cancer research laboratories (*3–30*).

The conversion of the solid hepatoma to an ascites form was undertaken chiefly to obtain a free tumor-cell population that could readily be sampled at intervals for studying the cytotoxic effects of body fluids of rats resistant to the growth of solid hepatoma (*2*). Also the extensive biochemical data that have been obtained with the solid tumor in different laboratories (*3, 5, 7, 9, 11, 12, 14–16, 18–30*) could perhaps be assessed more accurately with an ascites tumor, in which there is no stroma, and the relatively few nontumorous contaminating cells can be counted readily.

MATERIALS AND METHODS

Conversion and transplantation technique

The animals in this experiment were female Sprague-Dawley rats, weighing from 125 to 150 gm.

The technique for transforming the solid Novikoff hepatoma into an ascites tumor was basically that used by Lowenthal and Jahn (*31*) and subsequent workers (*32–35*).

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[1] Received for publication November 10, 1959.

[2] A preliminary report was read before the American Association for Cancer Research (*1*).

[3] Research carried out during the tenure of a Special Research Fellowship from the National Cancer Institute, National Institutes of Health, Public Health Service.

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Hemorrhagic peritoneal fluid was obtained from a carrier of solid tumor on the 5th day after intraperitoneal transplantation of a standard dose (*2*). This fluid was centrifuged, and the sedimented tumorous and nontumorous cells were washed several times with sterile isotonic saline and then resuspended in saline. A sample was saved for microscopic examination, and 1.0 ml. of the suspension was inoculated intraperitoneally into another rat (*first transplant*). After 6 days this carrier died, and about an hour later its tumor-containing, hemorrhagic peritoneal fluid was prepared as before for transplantation (*second transplant*). This procedure was followed once more (*third transplant*). Thereafter the peritoneal fluid was not centrifuged, but whole peritoneal fluid was harvested 7 to 15 days after transplantation and 1 to 5 ml. inoculated intraperitoneally into each of 3 animals. By the seventeenth transplant generation, a free-cell ascites tumor was definitely established.

For the first 8 transplant generations of the ascites hepatoma the transplantation procedure consisted of transferring small doses (0.1–0.3 ml.) of ascitic fluid, which were harvested after short periods (2–3 days). Starting with the ninth transplant generation a standard technique was adopted: Larger transplant doses (1.0–1.5 ml.) were used and the ascites was taken for examination and transplantation at greater intervals (7–8 days). In a few instances, injections were made into the muscle of the hind leg, into subcutaneous tissue on the back, or into a tail vein.

A sample of each tumor harvest used for transplantation was smeared on a slide, fixed by formaldehyde vapor, and stained by hematoxylin and eosin. Routine histological preparations were also made of the omentum (when nodules of solid tumor were detected grossly), mediastinal lymph nodes, intramuscular and subcutaneous tumor masses, and lung (after intravenous injection).

RESULTS

Conversion Period

When autopsied, the first host resembled in all ways a typical carrier of the solid tumor (*2*). The tumor was located mainly in the greater omentum and exhibited lymphatic spread to the nodes at the hilum of the liver and in the anterior mediastinum. In addition there was approximately 9 ml. of hemorrhagic peritoneal fluid.

Starting with the second transplant generation, tumor growth was more diffuse. It frequently grew not only on the greater omentum but also on the mesentery, the parietal peritoneum of the body wall and diaphragm, and the pelvic organs.

Prior to the sixteenth generation, the ascitic fluid contained a few free tumor cells, some tumor-cell aggregates, and much blood. In the sixteenth generation, the ascitic fluid became thicker, less hemorrhagic, and contained many more tumor cells—almost all of which were free (fig. 1).

Early Ascites Tumor

The first 8 transplant generations of the true ascites tumor, *i.e.*, after the sixteenth generation, contained varying quantities of single tumor cells and tumor-cell aggregates. Progressively increasing numbers of tumor cells of each transplant generation grew in groups of 2 to more than 50 cells (fig. 2). In addition, the ascitic fluid tended to become more hemorrhagic and solid growths in the greater omentum tended to be more extensive. The relatively high proportion of islands to free cells was maintained as long as the transplantation technique of injecting small doses after short intervals was used.

In the ninth and subsequent transplant generations, which were transplanted by means of larger doses after longer periods of growth, the tumor-cell population at the time of harvesting consisted almost entirely of free cells.

To elucidate the conditions favoring growth of the tumor as single cells and multicellular groups, the following experiment was done on two occasions. Starting with the free-cell tumor, small transplant doses, 0.1 ml., were inoculated after short intervals of growth. Transplantations were made for 6 consecutive generations, and each rat was tapped at 2- to 5-day intervals. For purposes of comparison, carriers of the single cell line—those transplanted after 7-day intervals with 1.0 ml. of tumor—were tapped periodically.

After 4 or 5 serial transplants of relatively small doses of the single-cell ascites tumor, made after short periods of growth, an ascites "island tumor" was derived; this could be maintained by using these standard transplant conditions (low doses and short intervals). The number of tumor cells growing in small groups of two or more cells increased in each successive transplant generation.

In any given transplant generation there was a progressive decrease in the number of multicellular groups in the later stages of growth, as well as a general decrease of the ratio of cells per unit volume of ascitic fluid. There was a suggestion of a direct relationship between the number of multicellular groups and the amount of blood in the ascitic fluid.

The mean survival time for the carriers of the ascites tumor during the first 8 generations was 8 days, excluding an occasional rat that survived for as long as 3 weeks. Autopsy of rats killed at various intervals after transplantation revealed no grossly observable solid tumor before the 4th day. However, on the 3d day, microscopic examination of sections of the greater omentum revealed one to several layers of tumor cells attached to the mesothelium but exhibiting only minimal infiltration of tumor cells beneath the serosa. In those rats that died spontaneously on or about the 8th day, a relatively large tumor mass, with focal areas of necrosis, involved the greater omentum. This resembled the postmortem appearance of animals carrying the solid form of the tumor. The few rats surviving until the 20th day or beyond had tumor not only in the greater omentum but also in the anterior superior mediastinal lymph nodes. These nodes were greatly enlarged and replaced by

metastatic tumor cells. In addition, hemorrhagic fluid, with a few tumor cells, was found in the pleural and pericardial cavities.

Established Free-Cell Ascites Tumor

The free-cell ascites tumor, tapped at varying intervals, contained several types of nontumorous cells in addition to various categories of tumorous cells (fig. 1). Generally, 24 hours after transplantation, the most numerous were the free tumor cells. In addition, the fluid contained a relatively large quantity of acute inflammatory cells (mainly eosinophilic leukocytes), some small multicellular tumor aggregates, and a few red blood cells. By the end of 4 days, the proportion of acute inflammatory cells markedly decreased, the multicellular tumor groups were rare, and red blood cells were numerous. At the time of transplantation (7–9 days), the concentration of tumor cells had decreased moderately and macrophages were relatively numerous. Many of the latter contained intact and fragmented red blood cells and what appeared to be nuclear debris. At this stage, as a consequence of a marked decrease in the proportion of red blood cells, the ascitic fluid became light pink. There were virtually no multicellular tumor units several days after transplantation. Preterminally, at about the 11th day, the fluid became markedly hemorrhagic again, and there was a decrease in the relative numbers of tumor cells.

The mean survival time of the carriers of the ascites tumor after the eighth transplant generation was 11 days.

Tumor carriers autopsied at various intervals presented a generally consistent pathological picture. Until the 4th or 5th day, there were no significant findings other than those already described. Microscopic study revealed tumor cells on and beneath the mesothelial surface of the greater omentum and, occasionally, relatively small quantities of hemorrhagic pleural and pericardial fluid containing a few tumor cells. When the latter finding was present, the anterior mediastinal lymph nodes were usually moderately enlarged and always at least partially replaced by tumor. In animals that died or were killed about the 11th day after transplantation, small macroscopically discernible tumor nodules were usually found within the greater omentum and there was some retroperitoneal spread around or near the left and occasionally the right adrenal gland. In addition, the anterior superior mediastinal lymph nodes were markedly enlarged and replaced by tumor. There was a relatively large volume of hemorrhagic fluid within the pleural and pericardial cavities. This contained a few tumor cells. Apparently the pleural fluid is a principal factor in the cause of death in most of the animals (presumably by compression, atelectasis, and suffocation). The mean survival time of the carriers of the later ascites tumor was 11 days.

After injection into the tail vein, multiple pulmonary metastases developed, but postpulmonary metastases were not observed in any of these animals. Inoculation of tumor into subcutaneous and intramuscular sites in 5 animals resulted in the progressive growth of a solid tumor mass

that killed 3 rats in 3 to 5 weeks. In the 2 remaining animals, after 2 and 3 weeks of progressive growth of the transplants which attained a diameter of 4 and 6 cm., the tumors regressed.

DISCUSSION

The relatively long latent period (16 transplant generations) required for conversion to the ascites tumor may be viewed in light of the investigations of the Kleins (*34*). From a study of a large number of transplantable tumors, they concluded that this period represents the time during which a selection of cells with characteristics best suited for ascites tumor growth takes place. The present observation of the continued gradual decrease in the quantity of solid tumor growth after the conversion to an ascites tumor may represent a further process of selection. The variation in number of islands and small multicellular groups of tumor cells with transplant dose and age of tumor may also reflect a similar process of selection among various stem lines.

The ascites tumor, like the solid form, shows only lymphatic spread. Tumor cells are found in the pleural cavities, mediastinal lymph nodes, and retroperitoneal space, but not in the lungs and abdominal viscera.

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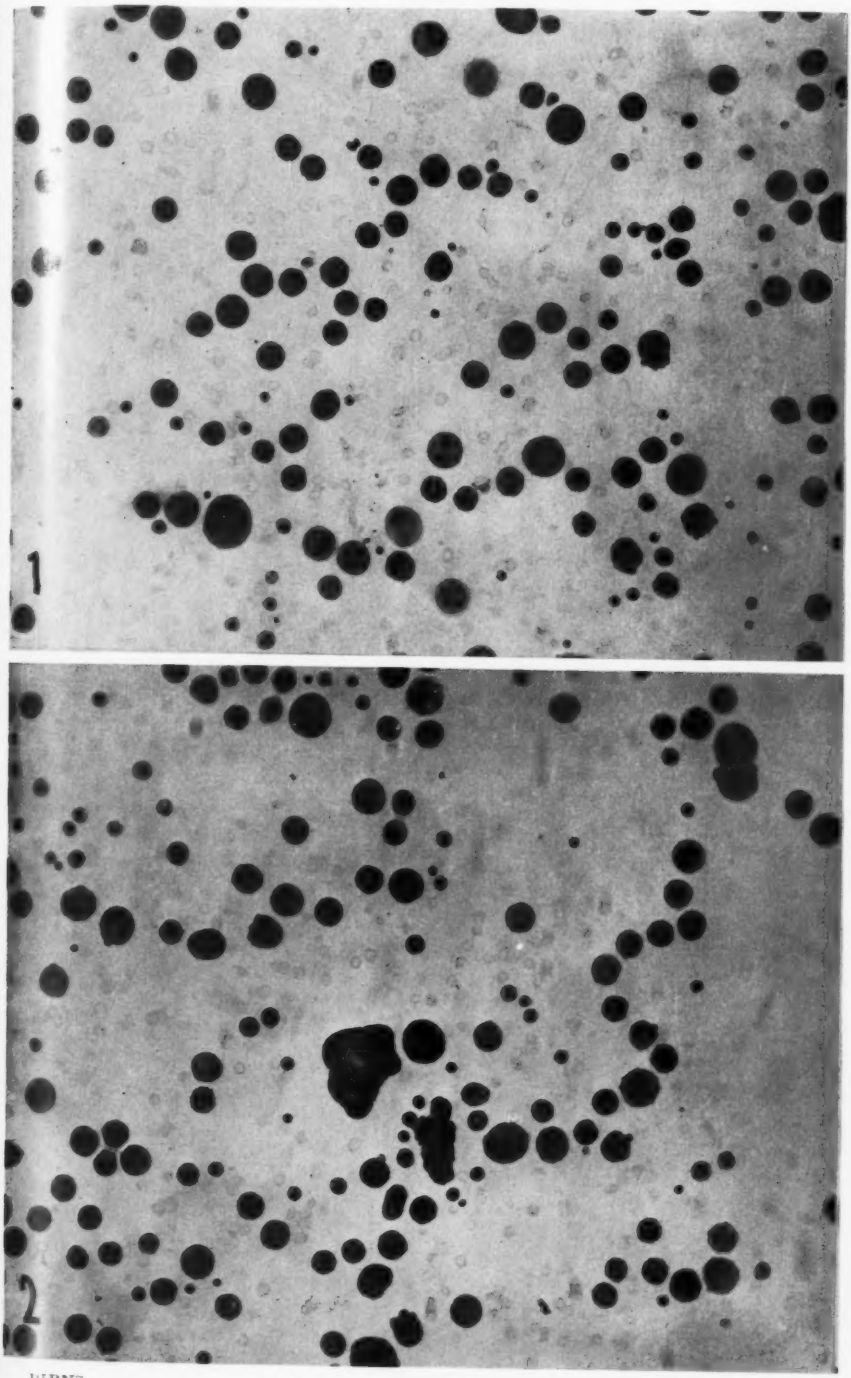
PLATE

PLATE 81

FIGURE 1.—Free-cell ascites hepatoma. Formaldehyde vapor fixation. Hematoxylin and eosin. × 315

FIGURE 2.—Island ascites subline. Smear contains mainly single cells as well as multicellular aggregates ranging from 2 cells to an island consisting of approximately 12 cells. Same fixation and stain. × 315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Hormone-Induced Tumors. I. Hamster Flank-Organ and Kidney Tumors In Vitro [1, 2]

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SUMMARY

Evidence derived from tissue-culture studies of an estrogen-induced dependent neoplasm of the kidney and an androgen-estrogen-induced dependent tumor of the flank organ in Syrian hamsters suggests that both are of epithelial origin. Cells from these hormone-dependent tumors can be grown in hormone-free media; the addition of crystalline hormone does not enhance growth or survival. However, when the kidney tumor is cultured as an "organ" fragment, growth does not occur, and survival time is greatly reduced unless estrogen is present in the culture medium. Responses to doses of 5, 10, and 25 μg. of diethylstilbestrol per ml. of culture medium indicate a graded effect—more mitoses occur at the higher value.—J. Nat. Cancer Inst. 25: 557–571, 1960.

SEVERAL YEARS ago, in the course of a broadly designed inquiry into the role of sex steroids as tumorigens in Syrian hamsters (*Mesocricetus auratus*) Matthews, Kirkman, and Bacon (*1*) and Kirkman and Bacon (*2–4*) observed that the prolonged administration of exogenous estrogen was followed by the appearance of a malignant renal neoplasm in males. Similar studies have been reported by Horning (*5*, *6*) and Horning and Whittick (*7*). Induction of this tumor was blocked by the addition of testosterone; however, when the effects of simultaneously administered estrogen and androgen were being investigated, it was observed that a tumor nearly always arose in the flank organ (*8*).[4]

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[1] Received for publication November 27, 1959.

[2] This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, and an American Cancer Society, Inc., Institutional Grant to Stanford University.

[3] The author wishes to express indebtedness to Professor Hadley Kirkman for his cooperation and excellent advice and to Professor Margaret R. Murray, in whose laboratory the author spent a year as a U.S.P.H. Fellow during the initial phase of this investigation. Appreciation is extended to my wife, Annette Algard, and to Mrs. Helen Lim, who have assisted in much of the actual work.

[4] Hamster flank organs are small, slightly raised, heavily pigmented structures, bilaterally situated in a costovertebral position. Although present at birth, they do not attain full size until sexual maturity. They are androgen-sensitive and most conspicuous in the sexually mature male. Histologically, they consist of many hypertrophied sebaceous glands and hair follicles interspersed with many melanocytes. They are thought to play a part in the mating prelude by producing some olfactory-stimulating substance. For a more detailed account, *see* descriptions of Lipkov (*9*) and Lochbrunner (*10*).

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Both of these tumors manifested complete dependence on the inducing agents. If exogenous hormones were withdrawn, the tumors ceased growing and showed signs of involution, more marked with the kidney neoplasm. Nor was subcutaneous transplantation successful without introduction of the appropriate hormones. After 14 generations the renal tumor acquired some degree of autonomy and, according to recent work by Kirkman (*11*), after being well started on exogenous estrogen, transplants were carried along on exogenous testosterone propionate alone, which is extremely interesting because primary inductions are completely blocked by this hormone. While it is true that some sublines of the renal tumor have achieved some autonomy, their propagation rates are relatively low and they do much better in host animals receiving stilbestrol, even after 4 years. Growth of the flank-organ tumor has remained completely dependent on the estrogen-androgen combination initially inducing it.

There has been wide disagreement among pathologists concerning the histogenesis and classification of these tumors, more so for the flank-organ tumor than for that of the kidney. Kirkman and Bacon (*12*) suggested that the kidney tumor might be a malignant adenoma but indicated that they believed significant contributions to the tumor came from interstitial connective tissue. Other possibilities, such as Wilms' tumor, were discussed by Kirkman and Robbins (*13*). Recently, based on histological studies, Horning and Whittick (*7*) have stated that this kidney tumor is a carcinoma of tubular origin. Kirkman (*13*) believes, ". . . it is certain that most of the tumor cells arise from tubular epithelium, the proximal convolution is definitely involved, but origins from distal segments are probable." However, he continues, ". . . a connective tissue contribution may occur also." He concludes, "Some skepticism is expressed concerning the adequacy of strictly morphological criteria in attempts to classify the tumor and to study its histogenesis." The flank-organ tumor is even more enigmatic and has been classified tentatively by various workers as neurilemmoma, neurofibroma, endothelioma, basal-cell epithelioma, and melanoma.

While much work has been done on the histology and endocrinological relationships of these tumors *in vivo*, it occurred to us that these studies might be augmented by the use of tissue-culture methods. Murray (*14*) and Murray and Stout (*15*, *16*) have demonstrated that it is sometimes possible to contribute a great deal of information on the cellular origin of certain tumors by comparing their cellular outgrowth with cells derived from suspected normal counterparts. Thus they have identified the Schwann cell as the origin of the neurilemmoma and have utilized tissue culture as a means of differentially diagnosing sympathicoblastomas, thymomas, lymphomas, and other tumors.

In addition to aiding in classification, we felt that tissue culture might provide a unique opportunity to resolve some endocrinological aspects of tumor dependence. Although there is considerable literature pertaining to the action of various hormones on normal and malignant cells and

on cultures of organs known to be hormone targets (*17*), as far as we know, hormone dependence of tumors *in vitro*, has not yet received the attention it deserves.

Whether hormone-dependent tumor cells actually require the inducing agent as a part of their nutritional environment or whether such dependency is secondary to general effects of the hormone is surprisingly difficult to assay in whole-animal experimentation. One would like to know, for example, which of several alternatives is best: Are the tumor cells metabolically altered so as to require relatively large amounts of hormones? If so, will this requirement be reflected by the tumor cells under culture conditions? Or, are the hormones acting at a supracellular level, inactivating "checks and balances" preventing tumor growth in the untreated animal? Regardless of the mode of operation, is it actually the hormone or some altered form of the hormone, *e.g.*, conjugated or degraded, that exerts this tumorigenic effect?

Obviously this list can be greatly expanded; however, one of the most provocative questions has arisen from a suggestion by Dr. H. B. Fell, that in addition to cell cultures, "organ cultures" of these tumors should be studied. Briefly this question concerns the possible effect of tissue organization on the relationship between the neoplastic cell and the hormone. As will be shown, data obtained from cell cultures are quite different from those obtained from "organ cultures" of the dependent renal tumor.

The purpose of this paper is to present our techniques of culture and some preliminary observations on the behavior of these hormone-dependent tumors under different conditions *in vitro*.

MATERIALS AND METHODS

Syrian hamsters bearing sex hormone-induced primary tumors of the kidney and flank organ or serial subcutaneous transplants of these tumors were obtained, with one exception, from the laboratory of Professor Hadley Kirkman, Department of Anatomy, Stanford University School of Medicine. On one occasion, a group of 5 animals bearing primary renal tumors was obtained from Miss Marilyn Robbins, Palo Alto Clinic Research Foundation, Palo Alto, California. Whenever present, metastases of these tumors, usually to the lungs, were used as culture material.

Information concerning the media used throughout this work is presented in the following summary:

1. Plasma, chick-embryo extract, human placental and other serums, and balanced salt solution (BSS) were prepared in our laboratory.
2. Beef serum ultrafiltrate and Eagle's basal medium were purchased from Microbiological Associates, Inc., Bethesda, Maryland.
3. Parker's defined medium 1066 was purchased from Connaught Laboratories, Toronto, Canada.
4. Diethylstilbestrol and testosterone propionate, as well as other sex steroids, were obtained through the courtesy of the Schering Corporation,

Bloomfield, New Jersey, or were purchased from Nutritional Biochemicals Corporation (NBC), Cleveland, Ohio.

5. Collagenase was obtained initially from E. Lasfargues, The Laboratory for Cell Physiology, College of Physicians and Surgeons, New York City. Subsequently, that obtainable from NBC was found to work equally well.

With the exception of our method for incorporating hormones into the culture medium and for a slight modification of Fell's organ-culture method, our techniques have not departed significantly from standard procedures described in various texts on tissue culture or in specific literature which will be cited.

A description of the most satisfactory method we have used for incorporating crystalline sex hormones into culture medium follows: The hormone is finely pulverized with mortar and pestle. A weighed amount of the powder is ground in BSS with a Teflon tissue grinder. The resultant rather coarse suspension is diluted to the desired concentration and shaken in a sonic oscillator (Raytheon Model DF 101, 250 watts) for 15 or 20 minutes at 10,000 cycles without the addition of a suspending agent. Stock suspensions of 1 mg. per ml. tend to settle out fairly rapidly and, unless stored in a refrigerator in tightly sealed vessels with a minimum of surface, tend to drift gradually into the acid *p*H range. However, mild agitation is sufficient to obtain a homogeneous resuspension and dilutions can be prepared accurately with little effort. Transillumination of dilute suspensions shows the particles to be extremely fine and of uniform distribution. One drop of a suspension containing sufficient hormone to bring the final concentration to the desired level is added to a mixture of equal portions of chick-embryo extract and cock plasma and stirred vigorously until the clot commences to set or is added to a liquid medium.

We have also dissolved the hormones in small quantities of 95 percent ethanol, which is then injected into relatively large amounts of BSS to produce a reasonably good microcrystalline suspension. Elias and Rivera (*18*) added hormones dissolved in absolute ethanol directly to medium 199, holding the alcohol concentration at 0.5 percent.

During the earlier phases of this work, tissues were grown in standard Maximov double-coverslip cultures or in roller tubes with the fragments embedded in clotted fowl plasma. As work progressed, the method of culture was modified to take advantage of the well-known fact that epithelium grows most typically on the surface rather than throughout the matrix of a thick plasma clot. Small tumor explants were simply placed on the surface of coverslips previously coated with a thin layer of plasma. Subsequently, tumor tissue was minced in a small quantity of BSS and subjected to digestion with collagenase, washed, and after final suspension, pipetted onto coverslips thinly coated with plasma. This "thin clot" technique, devised originally for the study of mammary-gland epithelium, and a comprehensive account of the use of collagenase in tissue culture have been presented at length by Lasfargues (*19*).

Organ cultures were of the type used by H. B. Fell, Strangeways Labora-

tory, Cambridge, England. They consist of small, randomly selected bits of tissue planted on the surface of a clot of fowl plasma and chick-embryo extract in a 60 mm. watchglass housed in a 110 mm. petri dish with a supporting ring of absorbent cotton saturated with water to insure high humidity.

An alternative method of culturing "organ fragments" has been developed in our laboratory. Three mm. glass beads are washed after being soaked overnight in concentrated nitric acid. Approximately 15 to 20 beads are placed in the bottom of a watchglass where they serve as a support for a small square of lens paper. Liquid medium, with or without hormone, is added to barely cover the surface of the beads and the tissue fragments are planted on the lens paper. This system has several advantages: (1) The difficulty frequently encountered in trying to float lens paper is obviated; (2) medium can be changed rapidly without disturbing the cultures; (3) the system is easy to clean. Capillary attraction between the beads is sufficient to insure that the lens paper remains moist.

Coverslip cultures were fixed routinely in absolute methanol and stained with Jenner-Giemsa; organ cultures were fixed in Bouin's fixative and stained with Harris' hematoxylin and eosin. Sections stained with hematoxylin and eosin can be assessed rapidly for tumor cell survival, since all nonliving portions exhibit a high degree of eosinophilia.

Since a single organ-culture experiment involves several manipulations of approximately 100 explants over a period of 1 month or more, great care must be taken to avoid any possible confusion. Four groups of culture dishes were prepared: I—nontreated controls, II—minimally treated with hormone, III—treated with intermediate amounts of hormone, and IV—maximally treated with hormone. Each group consisted of 4 culture dishes (1–4) containing 4 to 6 explants arranged in a sequence and assigned a permanent letter designation. Thus, for example, it was possible to identify a given explant as II3b or IV6d, etc. Each periodic sample contained 16 fragments. To facilitate histological processing, we constructed a small stainless-steel rack which accommodates 16 micro-Gooch crucibles. The assembly, containing the explants of one sample, was moved through a succession of petri dishes containing the fixative and various dehydrating agents and finally into paraffin for infiltration. To embed, fragments were pipetted from their respective crucibles and aligned according to designation in the embedding dish. All 16 explants were contained in the final trimmed block in such a fashion that the 4 comprising each series could be sectioned simultaneously. This procedure, with a little practice, requires little more time than that needed for a single piece of tissue.

OBSERVATIONS

Although these tumors have a stringent requirement for certain sex hormones *in vivo*, we have found that good outgrowth and cell survival,

even some growth, may be obtained readily in tissue culture in media containing little or no hormone (*20*).

Cells emigrate from plasma-embedded fragments within 48 to 60 hours after explantation. Typically, they form a fairly dense corona, more loosely arranged toward the periphery. Microscopically, the individual cells from both tumors bear some similarity to fibroblasts grown from subcutaneous areolar tissue; however, a conspicuous difference is the high degree of pleomorphism and nuclear aberrations of the tumor cell. Certain differences in behavior widen the gap between these tumor cells and normal fibroblasts. The tumor cells tend to cause much more lysis of the plasma clot and, as the culture ages, in the zone of outgrowth characteristic whorls appear—localized patches of more or less compactly arranged cells which show the isolated "holes" frequently seen in sheets of epithelium in culture (fig. 1). There is also a strong tendency for the individual cells to flatten, to show an increase in basophilia associated with fine cytoplasmic granules, and to undergo extensive nuclear budding. All degrees of this phenomenon may be found, from slight protuberances to completely separated "micronuclei." Giant cells are comparatively rare. It is difficult to form a very strong opinion of the tissue relationships of either the kidney or the flank-organ tumor in a 3-dimensional substratum, since both yield cellular outgrowths that have some characteristics of connective tissue and some of epithelium in culture.

When the culture conditions are modified so that cells lie on rather than within the clot, the characteristics of the outgrowth shift toward those of epithelium. This shift is enhanced by pretreating the tumors with collagenase; a suspension of the tumor provides small clusters of cells that quickly attach to the clot surface and form small "islands." This tendency is somewhat stronger in the kidney-tumor cultures. As may be noted in figures 2 and 3, such small sheets are decidedly epithelial in character.

When the best cellular sheets obtained from the kidney tumor are compared with sheets grown from similarly treated normal kidney, one does find many differences. The normal epithelium assumes a highly recognizable mosaic pattern with clearly delineated cell boundaries (fig. 4), while the kidney-tumor epithelium is more loosely knit and not nearly so homogeneous. In a comparison of the tumor cultures with cultures of the so-called mesoblastic epithelia, *e.g.*, serosa, lining of the ureter, vas deferens, a definite resemblance is easily noted. Although there is some variation in cell morphology, in no instance have we obtained from the kidney tumor more than one class of cells, with the exception of a few scattered macrophages and lymphocytes and occasional well-defined fibroblasts. This observation tends to negate the idea that the tumor is of twofold origin (*13*).

The flank-organ tumor in tissue culture behaves remarkably like the kidney tumor. When cultured the same way, it, too, tends to form islands or patches of cells that have a decidedly epithelial appearance. Certainly, tentative classifications of neurilemmoma, neurofibroma, and endothel-

ioma can be ruled out. The observation that lobes of the flank-organ tumor may sometimes be heavily pigmented and that slices of unpigmented lobes may give a positive reaction when incubated in a 3-(3,4-dihydroxyphenyl)alanine solution led us to attempt to promote melanin synthesis in tumor cultures by adding tyrosine, a melanin precursor, to the medium, and to attempt the demonstration of even small quantities of melanin with the silver staining techniques of Bielschowsky. The results from both attempts were negative. On the basis of our examinations of this tumor in tissue culture, we are inclined to regard it as being of epithelial origin; the suggestions that it originates from pigment cells, Schwann cells, or connective-tissue cells have not been supported by our studies. Long ago, Kirkman considered the flank-organ tumor to be epithelial, possibly arising from cells of the base of the hair follicle, a view which is certainly not ruled out by our observations.

When it was first observed that these dependent tumors grew well in a culture medium containing human placental serum, it was suspected that the sex-hormone content of the serum was high enough to provide the necessary requirement for survival and some growth, particularly of the kidney tumor, which requires only estrogen *in vivo*. It was more surprising to observe comparable behavior of explanted flank-organ tumor, since it seems unlikely that placental serum would contain much of the androgenic material necessary for the growth of this tumor *in vivo*. To test the possibility that growth or survival time, in culture, could be increased or decreased by manipulating the amount of estrogen in the medium, kidney-tumor cultures were prepared with a variety of media, with the following results:

(1) Chemically defined media alone produced good initial outgrowth containing an occasional mitotic figure, followed by rapid deterioration within 10 to 12 days.

(2) Chemically defined media plus 10 percent horse serum produced good initial outgrowth, moderate growth, and no deterioration in 10 to 12 days.

(3) Chemically defined media plus 10 or 20 percent serum from estrogenized tumor-bearing hamsters gave essentially the same results obtained with horse-serum-supplemented medium (2).

(4) Chemically defined media plus 10 percent serum from castrated hamsters produced good initial outgrowth, some growth, and no deterioration within 10 to 12 days.

(5) Chemically defined media plus 0.1, 0.5, and 1.0 μg. of estrogen produced results essentially similar to those stated in (1).

(6) Chemically defined media plus 5, 10, 20, 30, and 50 percent human placental serum produced effects essentially similar to each other and to those described in (1) to (5), with the exception that 50 percent serum-supplemented medium produced fairly rapid deterioration, usually within 1 week.

It would appear, then, that the behavior of cells cultured from the dependent renal neoplasm is not influenced, at least not recognizably,

by the level of the estrogen in the medium. Since serum from castrated hamsters greatly improved survival, it seems unlikely that lack of estrogen causes the deterioration noted in the chemically defined medium alone. Probably, undernourishment, excessive alkalinity, and toxicities that are neutralized by added serum contribute to the generally poor results reported in (1) and (5).

Thus far, similar testing of the flank-organ tumor has been frustrated by the fact that the testosterone available to us is severely toxic, even in minute quantities. Estrogen alone has no apparent effect on flank-organ tumor cells in culture.

During the past year, both renal and flank-organ tumors have been grown in the "organ-type" cultures described. More attention has been given to the renal tumor. Preliminary screening revealed that doses of stilbestrol as high as those of testosterone propionate used by Lasnitzki to obtain stimulatory effects on the connective tissue of human prostate gland (50 μg./ml.) or higher (*21*) destroyed our cultures within 1 week. However, in series treated with 5, 10, and 25 μg. of the hormone per ml. of culture medium, we have been able to demonstrate a degree of dependence *in vitro* approaching that obtained in animal experimentation.

As indicated in the section on materials and methods, random samples of explants prepared from any given tumor were selected for fixation. Since it is virtually impossible to avoid taking some necrotic tissue, one must estimate the amount as closely as possible. Analysis of the fixed material indicates that 1 fragment in 4 contains few or no living cells. On this basis, we have discounted 25 percent of the very poor or nonliving explants seen in the various samples taken at intervals during the experiment unless it was perfectly obvious that good surface healing had taken place.

On the 5th day, the time of the first sampling, most of the fragments from nontreated cultures contained some tumor tissue that looked normal, though areas of complete or partial necrosis were prevalent. There is a tendency for the healthiest tissue to form a "cortical zone," usually thicker on the side adjacent to the clot. However, this is also generally true of cultures under hormone treatment and is probably related to the availability of nutriment, oxygen, etc. Even in the healthiest looking masses, mitosis has not been seen. Subsequent samples taken during the 2d, 3d, and 4th weeks appeared progressively poorer, and no tumor tissue survived at the last sampling. In one experiment, all nontreated cultures were dead within 2 weeks. The fact that healing had taken place in more than 75 percent of the samples examined affords reassurance that we did not select an excessive number of necrotic fragments for the series receiving no hormone.

Figures 5, 6, 7, and 8 show representative sections of renal tumor after 13 days in organ culture.

Histologically, the 5-day samples from the cultures treated with 5 μg. of stilbestrol per ml. of medium resemble those receiving no hormone. All viable tissue was devoid of mitotic figures. Samples of this series taken

at the end of 2, 3, and 4 weeks contained viable tumor tissue interspersed as nodules, cords, and sheets among areas of partial or complete necrosis. In one instance, a 21-day-old culture contained several dividing cells.

In general, early samples from series treated with 10 μg. of hormone per ml. of medium appear to be in somewhat better condition than those typically present in the 5 μg. series. There is a sharper differentiation between the living and nonliving portions of the explants. Most striking, however, is the frequent occurrence of mitotic figures in all fragments. It is common to find 3 to 6 figures per 10 μ section. Subsequent samples are essentially similar to those taken at 5 days with the exception that mitotic figures are slightly more numerous—as many as 10 per 10 μ section.

The quantity of viable tumor tissue per explant is not appreciably greater in the series treated with 25 μg. of hormone per ml. of medium than in the 10 μg. series. This is surprising in view of the large number of mitoses one finds, even in the first sample. Thus far, cell divisions have occurred most abundantly during the 2d week, with as many as 42 figures counted in a single 10 μ section.

DISCUSSION

The study of tissue cultures of sex hormone-induced dependent neoplasms of the hamster kidney and flank organ has only partially resolved the question of their cellular origin. It has permitted the elimination of some possibilities suggested by routine pathological inspection. Thus, the flank-organ tumor is definitely not a neurilemmoma, neurofibroma, endothelioma, or sarcoma of any type. Our failure to demonstrate a positive DOPA reaction in cultured flank-organ tumor argues against its being a melanoma. From the nature of its outgrowth, we also know that the renal tumor is not myoepithelial in nature, thus ruling out the juxtaglomerular apparatus as the place of origin. It seems improbable that intrarenal connective tissue is its source. The epithelioid nature of the outgrowths from both of these tumors, especially when they are cultured by the "thin-clot" method of Lasfargues, suggests that they are of epithelial origin. Horning's view that the renal tumor is a carcinoma and Kirkman's belief that the flank-organ tumor arises from epithelium are supported by our observations.

Of greater interest and possible significance than the determination of the source of these hormone-dependent tumors is that the response of tumor tissue to the inducing hormones *in vitro* varies with the method of culture. We have never observed any effect of sex hormones on conventional cell cultures. Mitotic rate and survival time in culture seem to be independent of the hormones so important for their survival *in vivo*. Treatment with huge doses of estrogens seems to have no effect on cell cultures of the renal tumor; however, more than minute amounts of testosterone or testosterone propionate have proved quite toxic to the cultured flank-organ tumor.

In contrast to an essentially neutral response of cell cultures to the inducing hormones, "organ" cultures show a definite, fairly dramatic, positive response. Without the hormone there is comparatively poor survival and no mitosis. In hormone-treated cultures, survival is comparatively good and many mitoses occur. Moreover, this response appears to be graded—the maximal effect is obtained at concentrations of 25 μg. of stilbestrol per ml. of clotted medium.

That no clear effect can be obtained by treating cell cultures with crystalline hormone suggests that these hormones induce tumors by destroying or altering some control or complex of controls normally operative in growth regulation. Conversely, it would seem that the tumor cells do not have a nutritional requirement for the inducing hormones. However, our observation that survival and cell division are enhanced by administering the appropriate hormone to organ-fragment cultures of the renal tumor indicates that the hormone, as a tumorigenic agent, is operative at a local level, *i.e.*, the hormone in this instance is acting directly on the kidney or some portion of it, or if a tumor is present, on the tumor. The most obvious possibility is that the response to hormones is mediated in some way by a cellular or tissue interaction. Although the organization of the kidney tumor is relatively simple, it involves a characteristic connective-tissue stroma. Perhaps this stroma under influence of the hormone furnishes a factor, or complex of factors, required for tumor growth.

An alternative explanation for the difference in behavior of this renal tumor in "cell culture" and in "organ culture" must not be ignored. It is possible that cells comprising a dependent tumor may not be homogeneous with respect to their hormonal requirement and that some hormone-independent cells may be present in any sample of tissue placed in culture. It is possible to select for such an autonomous cell population in a hormone-free environment. In organ culture, where outgrowth is minimal or nonexistent, such a selection would be eliminated or at least would not be likely to manifest itself within the 1-month period of culture.

Reimplantation of cell cultures maintained in hormone-free medium into untreated host animals, if such selection occurs, should yield an autonomous version of the tumor. It would, of course, be important to avoid keeping such test cells in culture for more than relatively short periods to preclude the possibility of obtaining a completely "altered" population.

This experiment and others are now in progress.

CONCLUSIONS

1. An estrogen-induced dependent kidney neoplasm in the Syrian hamster appears in tissue culture to be epithelial, supporting suggestions that this tumor is a carcinoma.

2. An androgen-estrogen-induced dependent tumor of the hamster

flank organ, variously referred to as a neurilemmoma, endothelioma, neurofibroma, basal-cell epithelioma, and melanoma appears in tissue culture to be epithelial, thus favoring epithelioma.

3. Cells of these tumors in culture survive and exhibit limited growth in media devoid of hormones.

4. Addition of estrogens to cell cultures of the kidney tumor fails to enhance growth or survival.

5. "Organ" cultures of kidney tumor fail to grow and have a limited survival unless estrogen is added to the culture medium.

6. Addition of 5, 10, and 25 μg. of stilbestrol per ml. of culture medium enhances survival, and growth occurs in direct proportion to the size of the dose administered to organ cultures of renal tumor.

7. That hormone dependence occurs *in vitro* is interpreted to indicate that the hormone inducing and maintaining the kidney tumor *in vivo* acts directly on the kidney or its tumor and not via circuitous routes, *e.g.*, the pituitary gland or liver.

8. An alternative explanation of the difference in behavior of hormone-dependent tumors in cell and organ cultures is discussed.

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PLATE 82

FIGURE 1.—Epithelial outgrowth from flank-organ tumor after 10 days in culture. × 250

FIGURE 2.—Epithelial sheet grown from renal tumor after 11 days in culture. × 90

FIGURE 3.—Portion of epithelial outgrowth from kidney tumor after 11 days in culture. × 250

FIGURE 4.—Portion of epithelial outgrowth from normal adult kidney after 10 days in culture. × 300

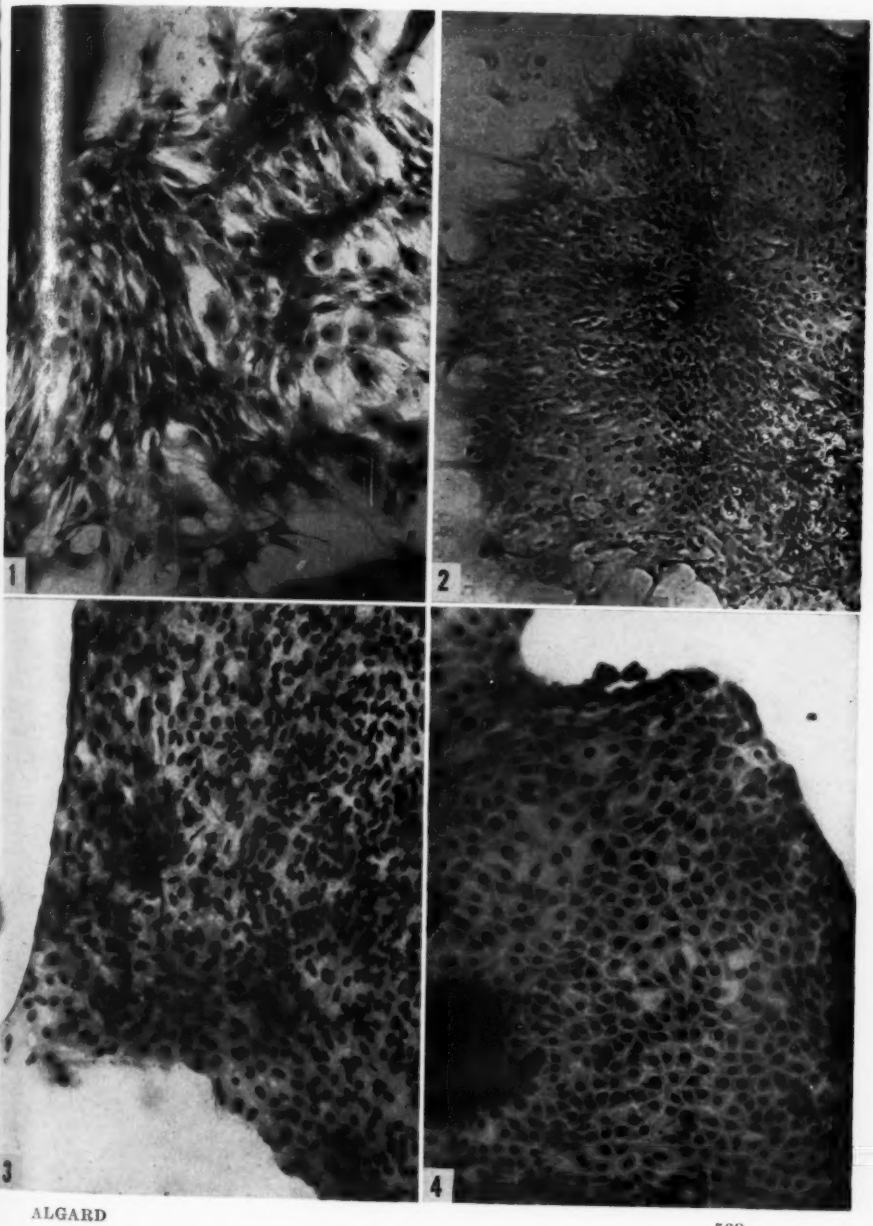
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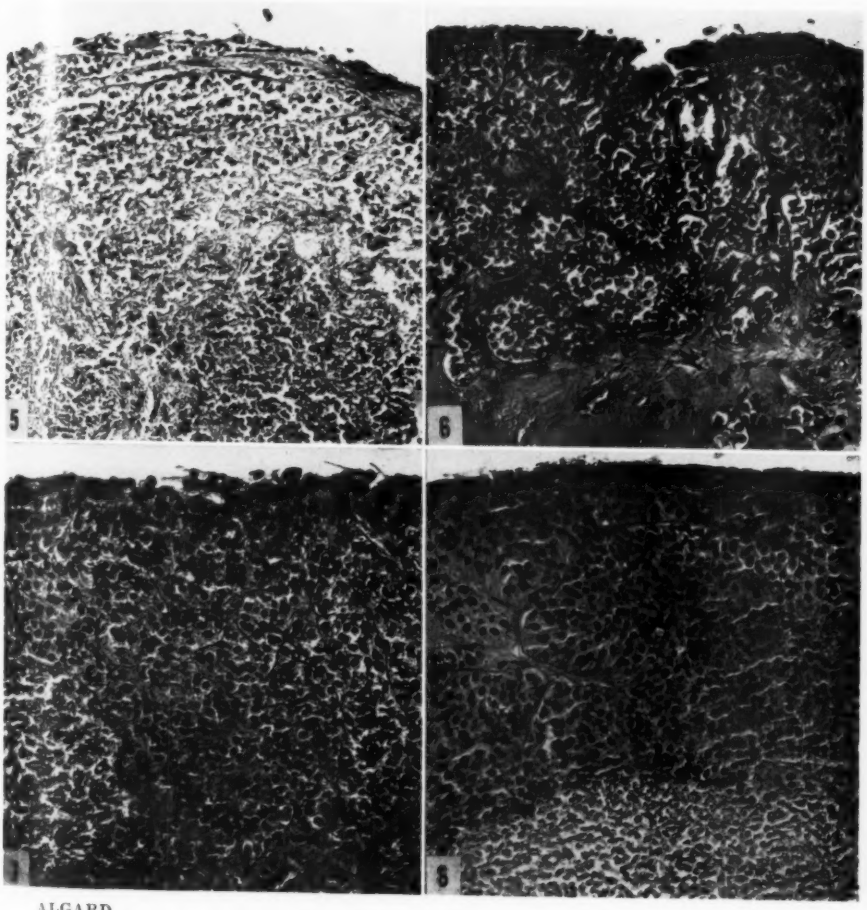
PLATE 83

FIGURE 5.—Renal-tumor organ culture after 13 days without estrogen. × 250

FIGURE 6.—Renal-tumor organ culture after 13 days with 5 μg. diethylstilbestrol per ml.of medium. × 250

FIGURE 7.—Renal-tumor organ culture after 13 days with 10 μg. diethylstilbestrol per ml. of medium. × 250

FIGURE 8.—Renal-tumor organ culture after 13 days with 25 μg. diethylstilbestrol per ml. of medium. × 250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Histopathology of Tumors Induced in Mice Treated With Polyoxyethylene Sorbitan Monostearate [1, 2]

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SUMMARY

A morphological study is presented of different types of skin tumors induced after repeated applications of polyoxyethylene sorbitan monostearate under various conditions. The tumors have been classified as papillomas, acanthomas, keratoacanthomas, benign adnexal tumors, squamous-cell carcinomas, and basal-cell carcinomas. Particular attention has been paid to the papillomas that regress; a series of papillomas studied before the time of maximum regression exhibited inflammatory changes that are described.—J. Nat. Cancer Inst. 25: 573–605, 1960.

A PREVIOUS paper (*1*) has described the induction of tumors in the skin of mice treated with polyoxyethylene sorbitan monostearate (Tween 60) alone or after a single dose of a carcinogenic hydrocarbon. The life history of these tumors has been described in detail and it has been noted that a large proportion of the skin lesions eventually regressed, even though treatment was continued. The present paper is concerned with the histopathology of the tumors induced in this study; the tumors that persisted to the death of the animals in some groups of the previous study will be considered. However, since many of the tumors occurring in such studies regress at an early stage, an additional experiment was undertaken to study the histology of these tumors which might have been expected to regress. In this group all tumor-bearing animals were killed after 20 weeks of treatment with Tween 60.

Although tumors induced in the skin of mice and rabbits by coal tar and pure chemical carcinogens have been described in detail (*2–10*), morphological descriptions of tumors induced after initiation and promotion in mice have been only briefly discussed (*11*, *12*).

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[1] Received for publication February 23, 1960.
[2] This investigation was supported by grant CS–9212, National Cancer Institute, National Institutes of Health, Public Health Service.

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MATERIALS AND METHODS

The experimental details have been given in a previous paper (*1*). In this study the skin lesions observed in the experiments in which Swiss mice were given Tween 60 daily or twice weekly with or without a prior single dose of 7,12-dimethylbenz[*a*]anthracene (DMBA) were included. Only the experiments in which DMBA was given 1 week prior to treatment with Tween 60 were considered, including the experiment in which the animals were caged individually. In addition, 30 female and 30 male 10-week-old Swiss mice were given a single dose of 20 μg. of DMBA followed, after an interval of 1 week, by daily treatment with 2 drops of Tween 60, for 20 weeks. At the end of the treatment, all the tumor-bearing animals, 18 females and 24 males, were killed.

Only the tumors available for detailed microscopic study were considered. Small tumors not clearly seen in the sections and tumors with severe autolytic changes were disregarded. A total of 413 tumors were available: 270 of them in experiments with DMBA followed by Tween 60 given for 50 to 80 weeks, 47 in experiments with Tween 60 alone, and 96 in the experiment with DMBA followed by Tween 60 for 20 weeks only. The skins were stretched on paper and fixed in 10 percent buffered formalin. One or more specimens from each tumor were taken, embedded in paraffin, and cut in sections of approximately 6 μg. Serial sections were often prepared and were stained routinely with hematoxylin and eosin and with special techniques when needed.

RESULTS

Morphological Considerations

Various classifications of experimentally induced skin tumors have been reported since the beginning of chemical carcinogenesis (*2–7*). The variety of terms suggested is due to the difficulty in drawing sharp lines between entities that tend to modify rapidly and that clearly progress from one type to another. In the present series of tumors, the same difficulties were encountered.

The following types of tumors were observed: papillomas, acanthomas, keratoacanthomas, squamous-cell carcinomas, benign adnexal tumors, and basal-cell carcinomas.

In addition, at all stages of the experiments hyperplasia of the epithelium and inflammatory changes of the dermis were observed. The most common type was a *simple hyperplasia*. It was usually diffuse, sometimes patchy, varied in degree, and was invariably associated with hyperkeratosis. The hair follicles were usually involved in the hyperplasia. Occasionally a *papillomatous hyperplasia* was seen, characterized by a distortion of the surface outline caused by high dermal papillae and hyperplastic hair follicles (fig. 1). In the living animals, the papillomatous hyperplasia appeared as a thick area with a granular surface, several

millimeters in size, distinguishable from a papilloma in that no circumscribed, elevated lesion was present. Usually these lesions remained unchanged for several weeks but eventually regressed. Sometimes small areas of the epithelium were involved in an *atypical hyperplasia* in which acanthotic and dyskeratotic changes were combined but with no invasion of the dermis or tumor formation. Small, intra-epithelial nests of basal cells were seen occasionally. In addition, one pseudoepitheliomatous hyperplasia was found at the margins of a chronic ulceration of the skin (fig. 18).

Papilloma

This benign tumor usually has been subdivided into pedunculated and sessile, mostly on a gross basis; microscopically the sessile papillomas have been further subdivided into two types (*7*). In this study the term *papilloma* was applied to all tumors in which a fibrous and an epithelial component were present in various proportions and in which the epithelium was hyperplastic but well differentiated (figs. 2, 3, 4, 5, and 6). The great majority of papillomas arose from the epidermis; some were associated with hyperplasia of the hair follicles and of the sebaceous glands. Occasionally, hair follicles included in the bulk of the tumors were cystically dilated and plugged with keratin. Some degree of thickening of the granular layer and hyperkeratosis were always present (fig. 5), and on occasion keratinization was prominent (figs. 4 and 6). The stalk of the papillomas often showed inflammatory changes; vessels were markedly dilated and congested, sometimes with thrombosis and hemorrhage (figs. 7 and 8). Frequently the epithelium was infiltrated by polymorphonuclear leukocytes (fig. 9) and was undergoing degenerative changes or even complete necrosis. Sometimes a papilloma seen grossly proved, on section, to be a mass of keratin and necrotic tissue (fig. 10); more frequently only the distal part was necrotic and the basal portion was better preserved (fig. 11). A hyaline change of the connective tissue of the tumor stalk was sometimes seen (figs. 12 and 13).

No significant morphological differences between the papillomas observed after 20 weeks of treatment and those in animals dying later in the experiments were noted. Although entirely necrotic papillomas were not seen in the 20-week study, the majority of the papillomas in this experiment showed inflammatory or vascular changes (figs. 8, 9, and 13).

Acanthoma

Lund (*13*) suggested the term *acanthoma* for benign epithelial tumors of the skin composed either of prickle or basal cells or both. We have used this term to indicate either tumors composed entirely of epithelial cells or papillomas in which there was an epithelial proliferation tending to grow into the dermis. Some atypical features of maturation of the epithelium, such as dyskeratosis or accumulation of basal cells, were often seen, but no rupture of the basement membrane or other histological features of malignancy were present (figs. 14, 15, 16, and 17). The gross appearance and the architecture of these tumors were either similar to

that of the papillomas, pedunculated or sessile (fig. 18), or assumed the shape of a broad-based, scaphoid tumor. The keratinization was usually moderate but occasionally prominent, particularly in the broad-based tumors (figs. 16 and 17). This type of tumor seems to include the "frill horns" on the ears of rabbits described by Rous and Kidd (*5, 6*), the second type of sessile papilloma and the conical tumors described by Shubik *et al.* in mice (*7*), and the keratoacanthoma type I described by Ghadially in rabbits, hamsters, and mice (*8, 9*). The acanthomas showed infiltration of inflammatory cells, dilatation of vessels, and hemorrhages—changes identical with those seen in the papillomas. Frequently there were small collections of pus which tended to separate the tumor from the dermis.

Keratoacanthoma

In human pathology this term is applied to a benign tumor characterized clinically by rapid growth and a tendency to regress, and morphologically by an active growth of well-differentiated nests of epithelial cells penetrating into the dermis. Tumors with similar morphological features have been repeatedly described in experimental conditions under different names (*5, 6, 8–11*) and attempts to establish analogies between the experimental and the human keratoacanthoma have been reported recently (*8, 9*). In this survey of tumors, such a histological pattern has been recognized on several occasions (figs. 19, 20, 21, 22, and 23). However, in the majority of cases a careful study showed focal areas of anaplasia, loss of cellular cohesiveness, cytological irregularities, and invasion of subcutaneous fat by individual atypical cells (fig. 24); therefore, they were considered squamous-cell carcinomas. Only in 3 instances was this not true (figs. 19, 20, and 21). Grossly these tumors reached dimensions of over 1 cm., were usually bud-shaped, deeply located in the skin, covered by keratin or necrotic tissue, and with indurated and elevated edges.

Squamous-Cell Carcinoma

In most instances the squamous-cell carcinomas were recognized on gross examination. Occasionally carcinomas appeared as small papillomatous lesions and their nature was recognized only at histological examination (figs. 25 and 26). In a few instances a lesion interpreted grossly as a simple ulceration proved microscopically to be an invasive anaplastic carcinoma. The possibility of the derivation of a squamous-cell carcinoma from a better differentiated penetrating tumor has been already mentioned. Several carcinomas arose from small, intradermal nodules which remained encapsulated for several weeks and finally ulcerated. Of a total of 72 squamous-cell carcinomas, 12 had metastases in the regional lymph nodes or in the lungs.

Benign Adnexal Tumors

In our series there were two types of benign adnexal tumors: One was a *cystic keratoma,* which was seen in 4 animals and consisted of single or

multiple epidermal cysts of evident follicular origin, containing abundant keratin. The epithelium was usually hyperplastic but sometimes thin because of compression (fig. 27). On one occasion rupture of the cyst wall with a foreign-body reaction to keratin had occurred. The second type of benign adnexal tumor was the *sebaceous adenoma* which had the gross appearance of a small, white, smooth sessile papilloma and was seen in 4 instances, 2 of them outside the painted area. On histological examination these tumors were composed of closely packed lobules of sebaceous glands, covered by hyperplastic epidermis (fig. 28). Papillomas with a hyperplastic component of sebaceous glands or hair follicles were not included in this group.

Basal-Cell Carcinoma

A high incidence of basal-cell carcinomas has been reported previously in rats treated with 2-anthramine or 3-methylcholanthrene (*14–17*). Merenmies (*12*) described basal-cell papillomas in mice treated with DMBA and Tween 60. In the present study 15 basal-cell carcinomas were observed in 12 mice, 3 and 2 being, respectively, in the same animal. Grossly they appeared either as papillomatous growth or erosions of the epidermis (figs. 29 and 30). They grew slowly and never reached more than a few millimeters in size. The tumors were composed of solid nests of basal cells growing deep in the dermis. Usually the tumor cells had scanty cytoplasm and an oval, deeply staining nucleus (fig. 31), but occasionally the cytoplasm was abundant and the nucleus showed irregularities in shape, size, and chromatic properties. In several cases sebaceous and squamous differentiation was seen (fig. 32). No metastases were observed.

Distribution of Types of Tumors

In the experiments considered for this morphological study, the total number of tumors available for a detailed histological study was approximately 65 percent of the total number of tumors present at the death of the animals. Ninety-six tumors were present in the animals killed after 20 weeks of daily treatment with Tween 60 after a single dose of DMBA. There were 77 papillomas, 14 acanthomas, and 5 basal-cell carcinomas. Many papillomas had edema and inflammatory infiltration of the stroma, dilatation and thrombosis of the blood vessels, and necrotic changes of the epithelium; those features were thought to be associated with the regression of the tumors. Two hundred and seventy were tumors studied from various groups given one initiating dose of DMBA followed by Tween 60 until the death of the animals. Of those, 132 were papillomas, 53 acanthomas, 3 keratoacanthomas, 66 squamous-cell carcinomas, 7 benign adnexal tumors, and 9 basal-cell carcinomas. Ninety-two percent of the acanthomas and 74 percent of the squamous-cell carcinomas were in mice dying after the 40th week of treatment, while the papillomas were homogeneously distributed through the different times of the experiments. Among the 47 tumors seen in the groups

given Tween 60 alone, there were 39 papillomas, 6 squamous-cell carcinomas, 1 acanthoma, and 1 basal-cell carcinoma.

DISCUSSION

The histological study of the skin tumors produced by repeated applications of Tween 60, with or without a previous dose of DMBA, revealed three types of benign epidermal tumors: papillomas, acanthomas, and keratoacanthomas. The essential feature of the acanthomas was an active, growing epithelium with some cytological and histological deviations from the normal, while the papillomas were fibroepithelial tumors in which the epithelium was hyperplastic but typical and well differentiated. The few keratoacanthomas could be a subtype of acanthomas, but were considered separately because of their invasive features and the gross and histological difficulties in distinguishing them from squamous-cell carcinomas. No information could be obtained on their propensity to regress. It is interesting to note that under present experimental conditions several squamous-cell carcinomas appeared to arise from keratoacanthomas.

Until recently, basal-cell carcinomas have been reported to have been induced only in rats (*14–17*). In the present study these tumors were induced in mice, though not frequently. Their characteristic low degree of malignancy, as in the human, was observed experimentally.

From the data presented in the preceding paper (*1*), it is evident that a large portion of the tumors induced by the sequency of DMBA-Tween 60 appeared during the first 20 to 30 weeks of experiment and rapidly regressed. To study the morphology of these tumors, an experiment was undertaken in which the tumor-bearing animals were killed after only 20 weeks of Tween 60 treatment. From this experiment it appears that the great majority of the tumors initially induced were papillomas, many of them with inflammatory changes. However, several acanthomas were also seen. In the experiments in which the treatment with Tween 60 lasted for a longer period, most of the acanthomas were seen in animals dying after the 40th week. Some of the acanthomas appeared to arise as such; others clearly began as acanthomatous areas in fibroepithelial papillomas. Histologically and cytologically the acanthomas, though some may regress, appear more likely to progress to squamous-cell carcinoma than the papillomas. This progression was demonstrated for the keratoacanthomas.

In mice treated with Tween 60 only, the same types of tumors were observed. The distribution was, however, different, the great majority being papillomas. In view of the over-all lower incidence of tumors and the longer latent period, these comparisons should not be emphasized. From this study it appears as though the results obtained were qualitatively similar in all experiments. On the basis of a morphological com-

parison, no distinction can be made between the action of Tween 60 alone or after administration of a more potent carcinogen.

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PLATE 84

FIGURE 1.—Mouse killed after 44 weeks of daily treatment with Tween 60, after single application of DMBA. Papillomatous hyperplasia: papillary distortion of external outline and elongation of rete pegs. Inflammation of dermis. Hematoxylin and eosin. × 45

FIGURE 2.—Mouse treated with Tween 60 daily for 56 weeks. Multiple well-developed papillomas. A total of 16 was recognized at autopsy. In each papilloma fibrous stalk is covered by hyperplastic epidermis. Hematoxylin and eosin. × 16

FIGURE 3.—Mouse treated with Tween 60 daily for 36 weeks, after single application of DMBA. Papilloma with broad stalk; hyperplasia of epidermis. Hematoxylin and eosin. × 25

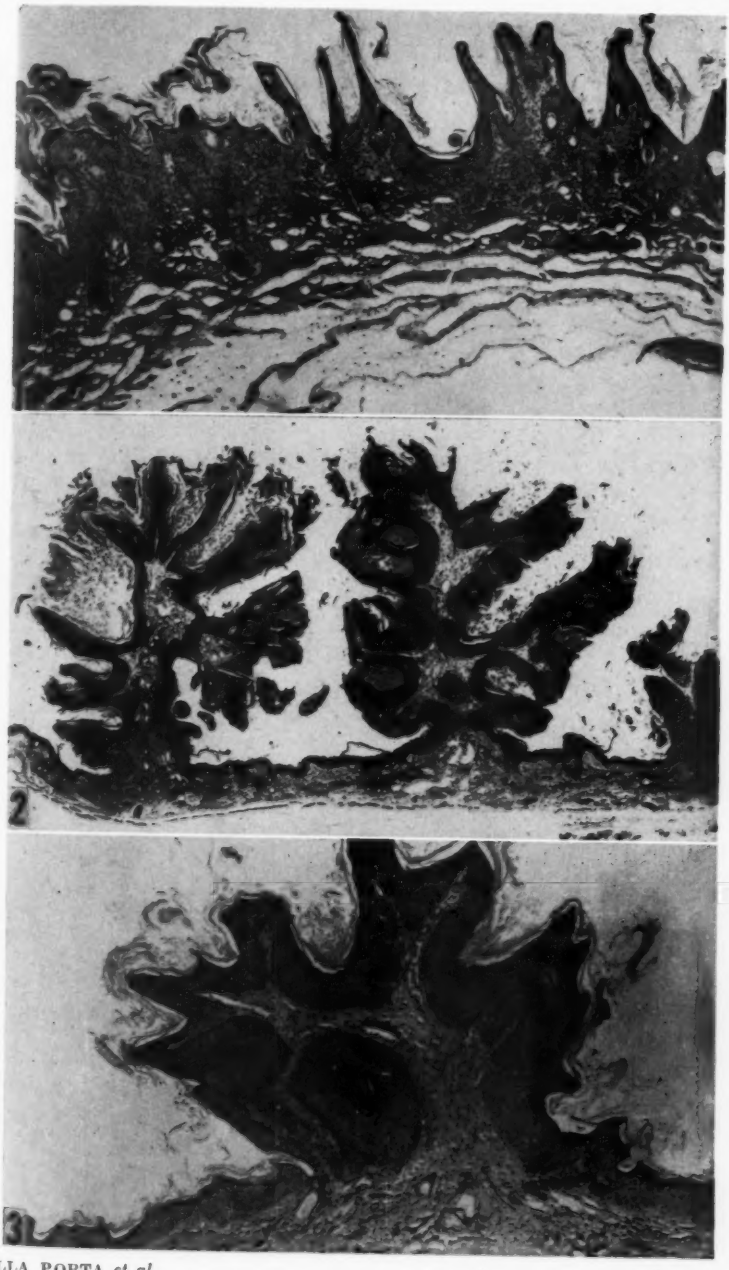

PLATE 85

FIGURE 4.—Mouse dying after 44 weeks of daily treatment with Tween 60, after single application of DMBA. Papilloma, hyperkeratotic. Central keratinized plug surrounded by fibroepithelial formations with hyperplasia of epidermis. Other 4 hyperkeratotic papillomas were seen in same animal. Hematoxylin and eosin. × 16

FIGURE 5.—Higher magnification of same tumor as in figure 4. Epidermis hyperplastic. Thickening of granular layer. Hematoxylin and eosin. × 400

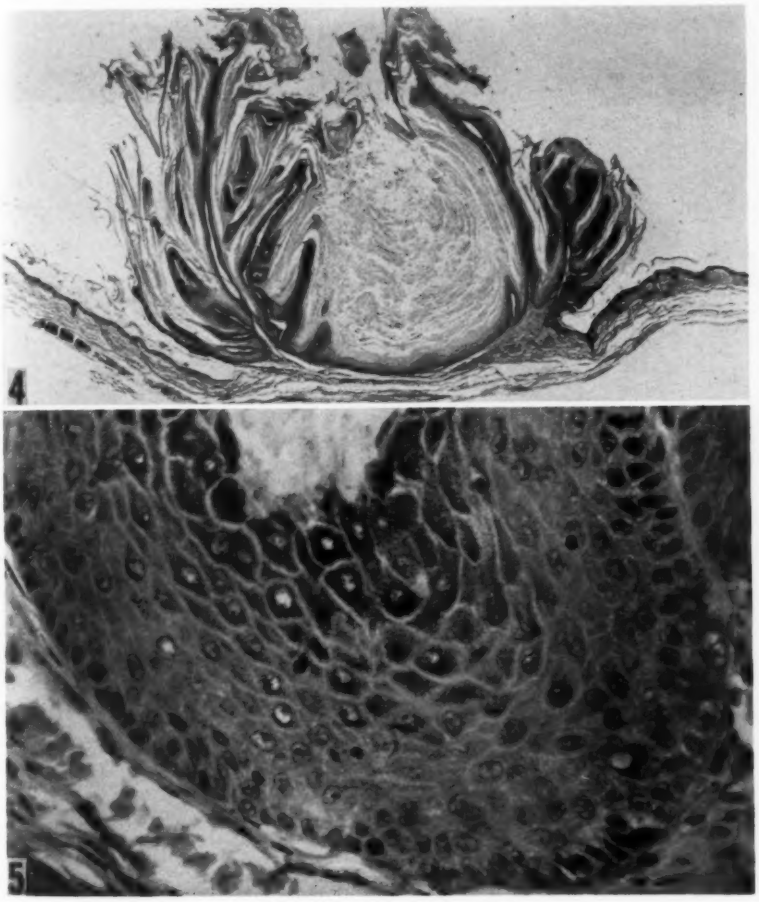

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PLATE 86

FIGURE 6.—Mouse dying after 45 weeks of daily treatment with Tween 60, after single application of DMBA. Papilloma, hyperkeratotic, pedunculated. Fibroepithelial formations partly surrounding large core of keratotic material which projects upward in cornu cutaneum-like fashion. Hematoxylin and eosin. × 22

FIGURE 7.—Animal painted with Tween 60 daily for 51 weeks, after single application of DMBA. Distal portion of papilloma, regressing: edema and hemorrhages in connective tissue. Hematoxylin and eosin. × 100

FIGURE 8.—Animal killed after 20 weeks of daily painting with Tween 60, after single application of DMBA. Papilloma, pedunculated. Dilatation of blood vessels and hemorrhages in stalk. Hematoxylin and eosin. × 38

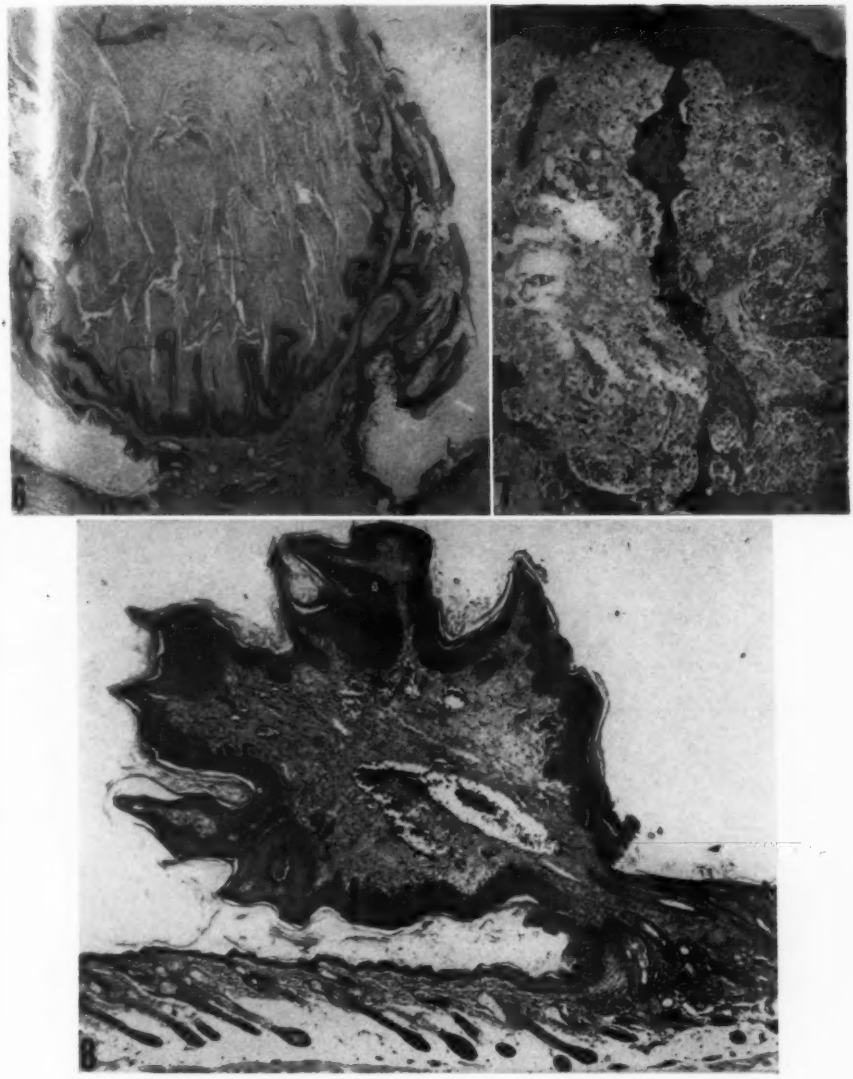

PLATE 87

FIGURE 9.—Papilloma, regressing, present in another animal killed at termination of 20-week experiment. Infiltration of inflammatory cells in connective tissue and among epidermal cells. Hematoxylin and eosin. × 300

FIGURE 10.—Papilloma, regressing, in animal killed at 74th week of treatment with Tween 60, after single application of DMBA. Central part of tumor is composed of necrotic tissue and keratin, while fibroepithelial formations are still recognizable in peripheral part. Hematoxylin and eosin. × 28

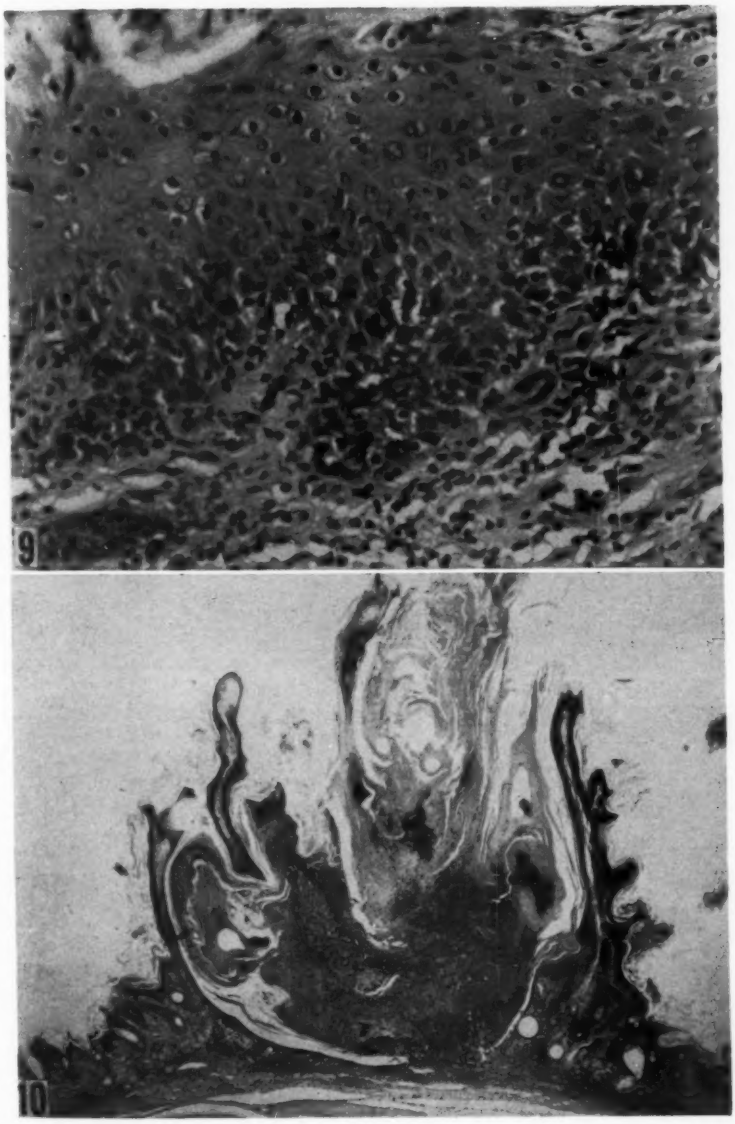
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PLATE 88

FIGURE 11.—Animal killed after 75 weeks of daily treatment with Tween 60, after single application of DMBA. Distal portion is composed of keratin and necrotic tissue, while at base the dermis is heavily infiltrated by inflammatory cells. Hematoxylin and eosin. × 40

FIGURE 12.—Distal part of regressing papilloma in animal dying after 41 weeks of daily treatment with Tween 60, after single application of DMBA. Hyaline changes and dilatation of blood vessels in stalk. Hematoxylin and eosin. × 100

FIGURE 13.—Papilloma, sessile, in animal killed at end of 20-week experiment. Hyaline changes centrally in stalk. Hematoxylin and eosin. × 38

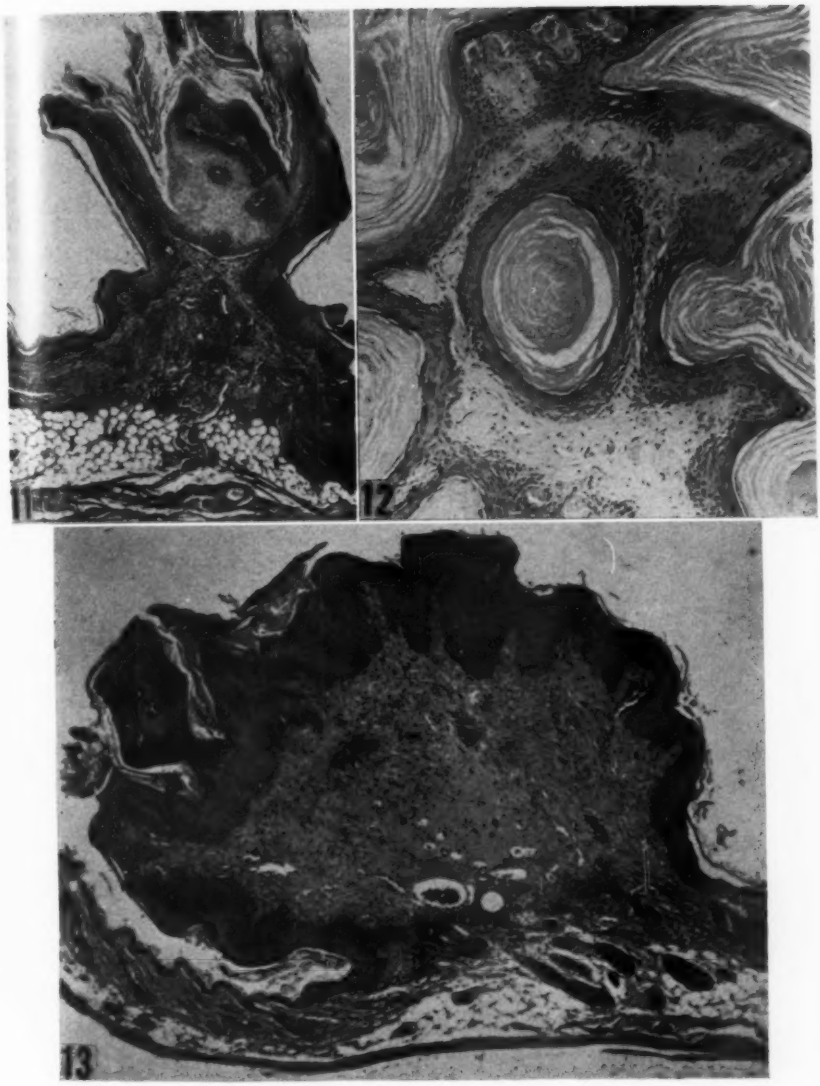
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PLATE 89

FIGURE 14.—Acanthoma in animal dying at 62d week of treatment with Tween 60, after single application of DMBA. This tumor was present for 8 weeks and had been grossly recognized as a sessile papilloma. Proliferation of epidermal cells growing downward with incomplete focal keratinization. Hematoxylin and eosin. × 100

FIGURE 15.—Animal treated with Tween 60 for 61 weeks, after single application of DMBA. Acanthoma composed of bundles of prickle cells growing downward in the dermis. Some mitoses are present. Hematoxylin and eosin. × 100

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PLATE 90

FIGURE 16.—Acanthoma, hyperkeratotic, in animal dying after 68 weeks of treatment with Tween 60, after single application of DMBA. This lesion had been grossly recognizable since 20th week of treatment. Scaphoid-shaped tumor composed of hyperplastic epithelium around central big plug of keratin. Hematoxylin and eosin. × 16

FIGURE 17.—Acanthoma, hyperkeratotic, in animal dying at 76th week of daily treatment with Tween 60, after single application of DMBA. This tumor had appeared 12 weeks before death. Hematoxylin and eosin. × 25

FIGURE 18.—Pseudoepitheliomatous hyperplasia in animal dying after 82 weeks of daily treatment with Tween 60, after single application of DMBA. On gross examination since the 56th week, small crusty ulcer had been recognizable. Ulcer of skin and growth of well-differentiated epithelial nests into granulation tissue. Hematoxylin and eosin. × 100

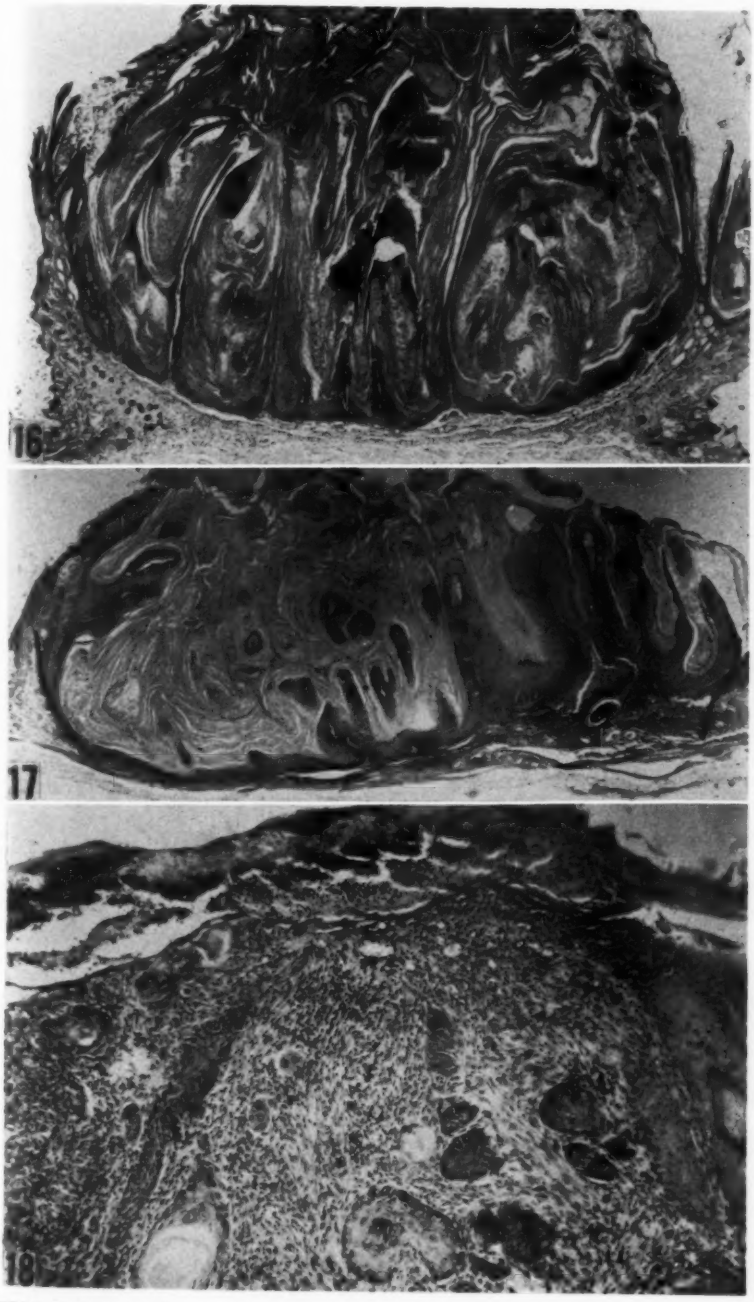
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PLATE 91

FIGURE 19.—Keratoacanthoma in animal painted with Tween 60 twice weekly, after single application of DMBA, killed at 51st week of treatment. This tumor arose at 43d week of treatment and grew rapidly. Proliferation of epidermal nests heavily keratinized, penetrating into dermis. At one edge skin is ulcerated. Hematoxylin and eosin. × 14

FIGURE 20.—Same tumor of figure 19. Penetrating nests are composed of well-differentiated epithelium. Inflammatory infiltration in dermis. Hematoxylin and eosin. × 180

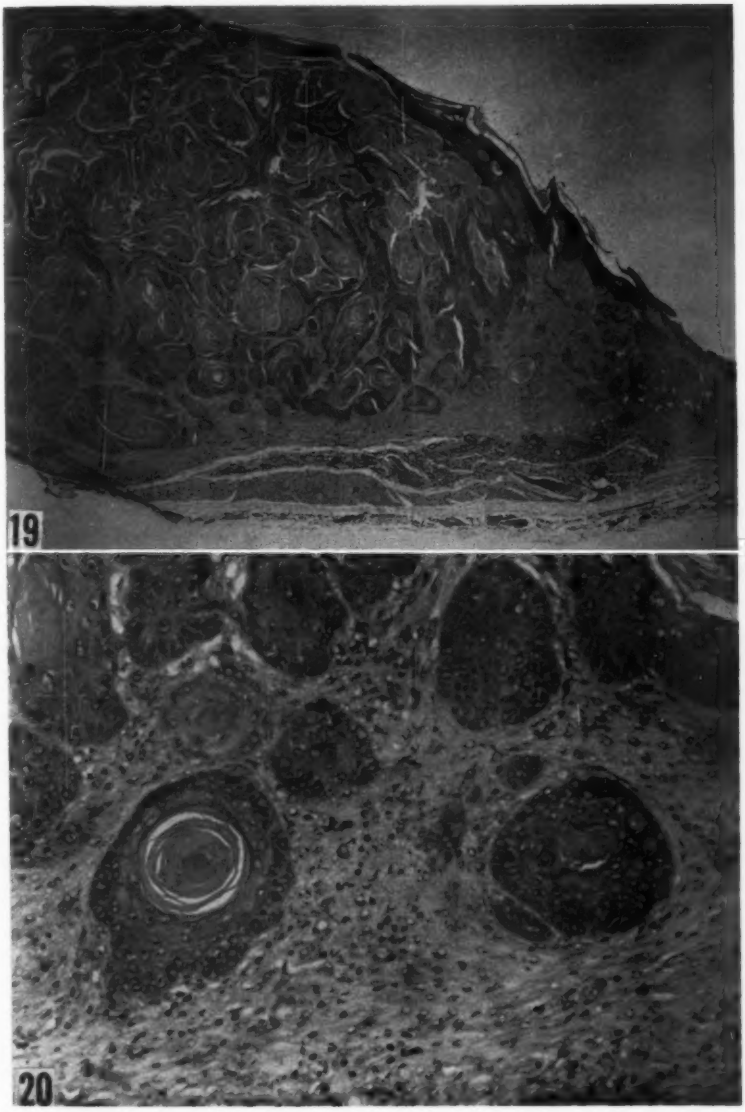
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PLATE 92

FIGURE 21.—Keratoacanthoma on ear of animal painted with Tween 60 daily for 62 weeks, after single application of DMBA. Tumor arose at 46th week as small papilloma. Hematoxylin and eosin. × 15

FIGURE 22.—Big, penetrating scaphoid tumor present in animal killed at 53d week of daily treatment with Tween 60, after single application of DMBA. Tumor had been present since 33d week but it had been preceded by other papillomas, in same area, that eventually regressed. General architecture is undistinguishable from keratoacanthoma. Hematoxylin and eosin. × 7

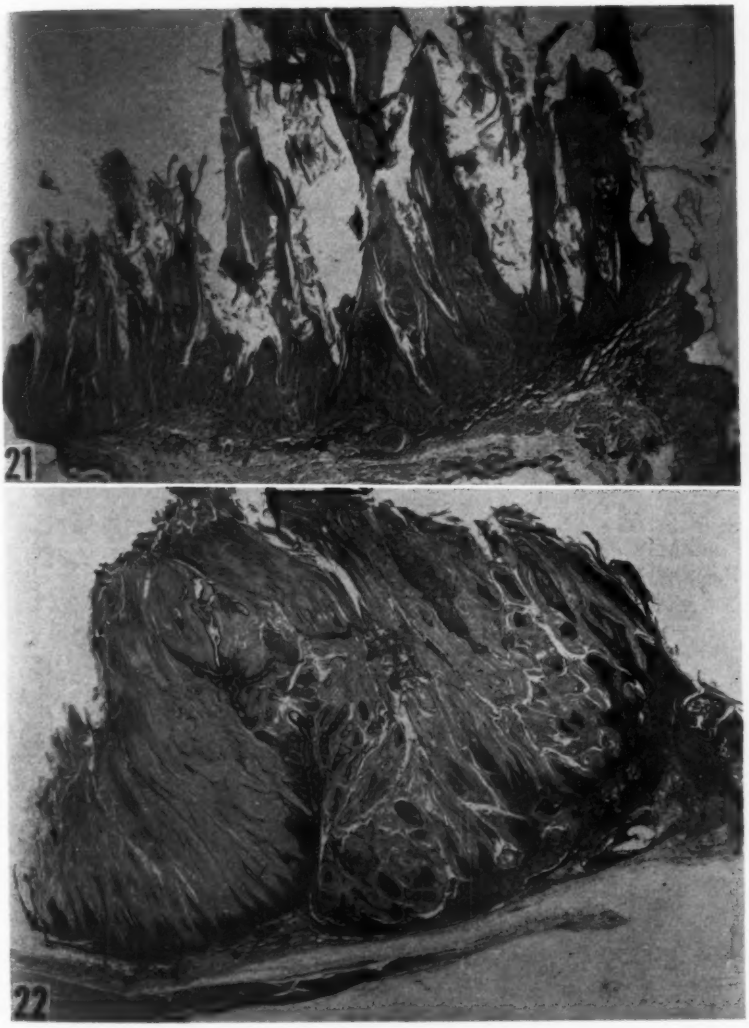
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PLATE 93

FIGURE 23.—Same tumor as in figure 22. In this area proliferation is composed of well-differentiated epithelium. Hematoxylin and eosin. × 120

FIGURE 24.—Same tumor as in figures 22 and 23. Carcinomatous nature of lesion was evident in subcutaneous fat where invasion by atypical cells could be demonstrated. Hematoxylin and eosin. × 180

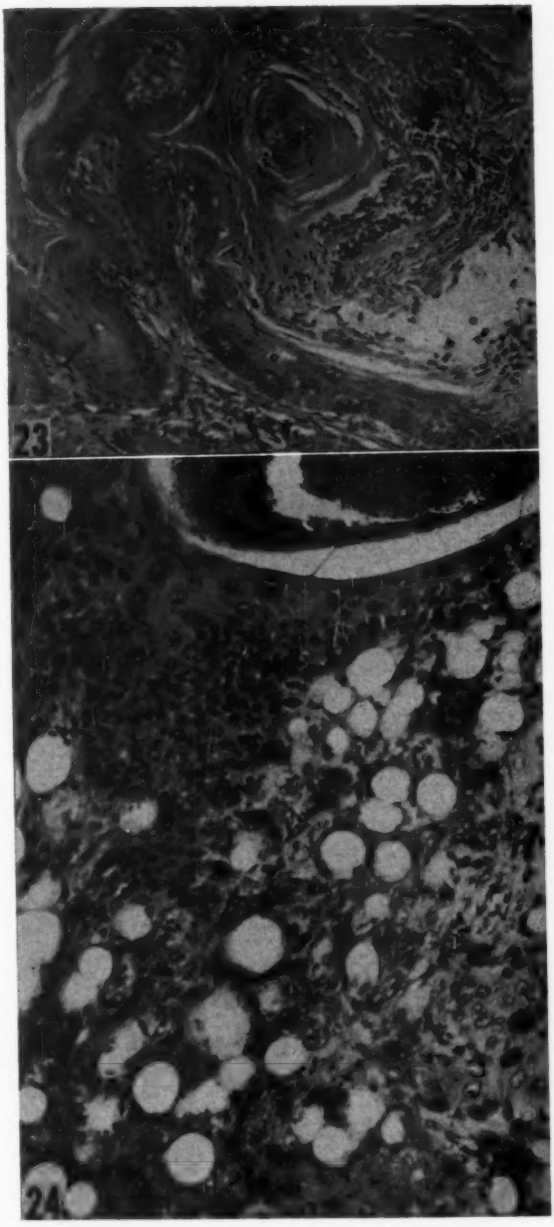

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PLATE 94

FIGURE 25.—Animal painted with Tween 60 for 59 weeks, after single application of DMBA. Squamous-cell carcinoma, recognized at histological examination in lesion present for 10 weeks, grossly considered as papilloma. Hematoxylin and eosin. × 45

FIGURE 26.—Higher magnification of same tumor shown in figure 27. Cellular disarray and atypicalities at base of tumor. Hematoxylin and eosin. × 300

FIGURE 27.—Animal dying after 69 weeks of treatment with Tween 60, after single application of DMBA. Cystic keratoma composed of multiple epidermal cysts containing keratin, in subcutaneous tissue, lined by thin epithelial layer. Hematoxylin and eosin. × 25

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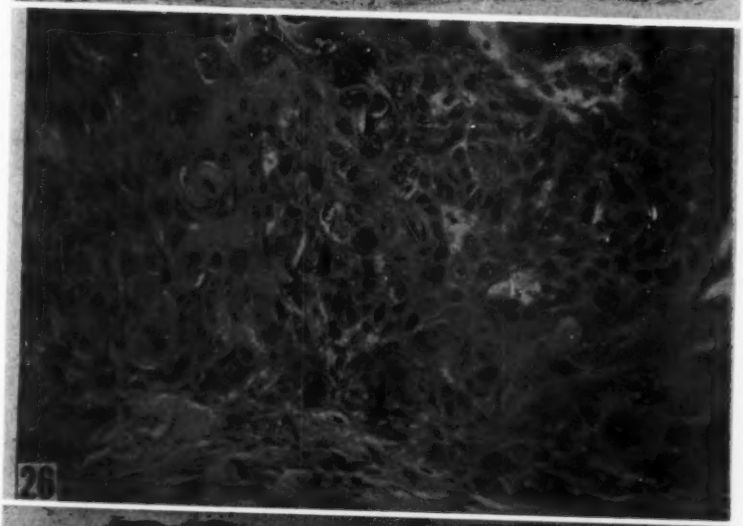
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PLATE 95

FIGURE 28.—Sebaceous adenoma in animal treated with single application of DMBA prior to daily treatment with Tween 60 for 50 weeks and killed 9 weeks after end of treatment. One sessile papilloma in right flank had been present for 10 weeks. Tumor is composed of lobules of sebaceous cells. Hematoxylin and eosin. × 50

FIGURE 29.—Animal killed at end of 20-week experiment. Basal-cell carcinoma in lesion grossly considered as papilloma. Hematoxylin and eosin. × 25

FIGURE 30.—Animal painted with Tween 60 twice weekly for 73 weeks, after single application of DMBA. Small crust had been present for several weeks. Basal-cell carcinoma. Hematoxylin and eosin. × 95

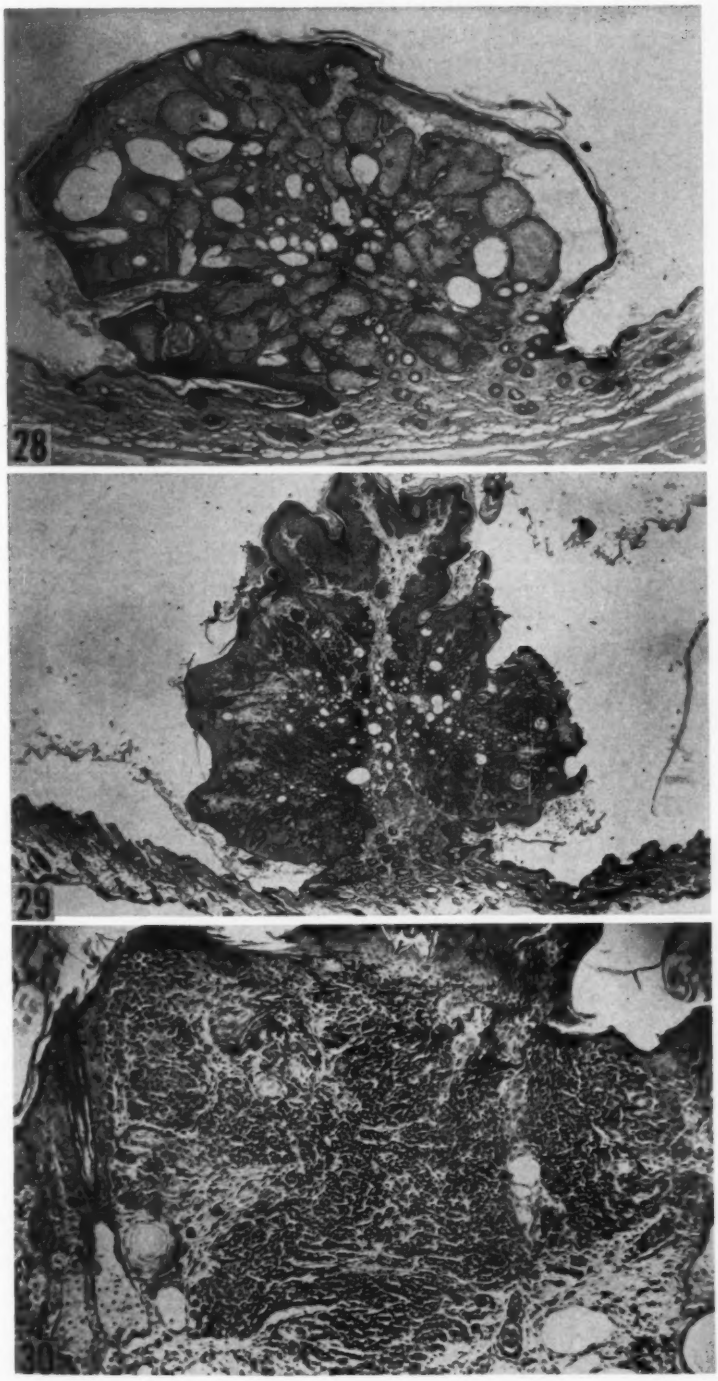
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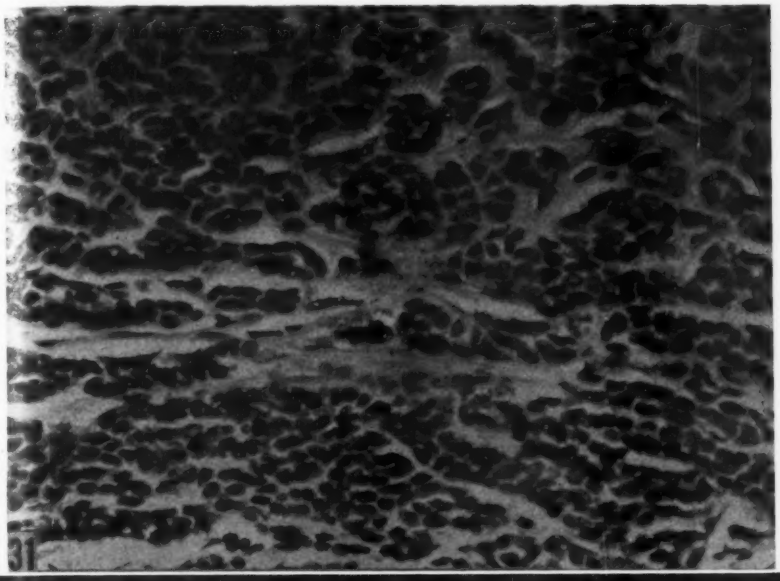
PLATE 96

FIGURE 31.—Same tumor as in figure 32. Irregular proliferation of basal cells, with scanty cytoplasm and round-oval, deeply staining nucleus. Hematoxylin and eosin. × 300

FIGURE 32.—Animal killed after 61 weeks of daily treatment with Tween 60. Tumor present on face for 2 weeks. Basal-cell carcinoma with sebaceous and squamous differentiation. Hematoxylin and eosin. × 330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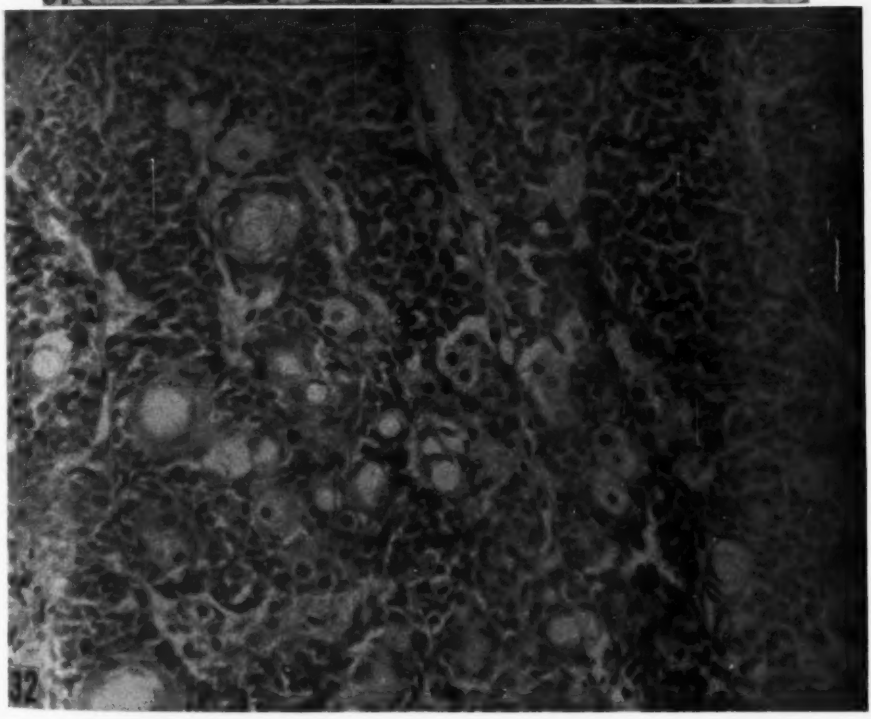
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Role of Polyoxyethylene Sorbitan Monostearate in Skin Carcinogenesis in Mice [1,2,3]

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SUMMARY

A detailed record of tumors induced in mouse skin by repeated applications of polyoxyethylene sorbitan monostearate (Tween 60) with and without a prior single application of a carcinogenic hydrocarbon is reported. Most tumors induced subsequently regressed. Only a small proportion, approximately 3 percent, progressed to carcinomas. Tween 60, at a dose of 60 mg. administered twice weekly or daily, was effective as a promoting agent. When given alone at the same dosage, Tween 60 gave rise to an appreciable number of tumors, with a far longer latent period. The effects of dosage, different initiating agents, the interval between initiation and the Tween applications, and the individual caging of animals are described. The nature of the activity of Tween 60 is compared with croton oil and discussed.—J. Nat. Cancer Inst. 25: 607–625, 1960.

THE PHENOMENON of promotion in skin carcinogenesis was described in the mouse when a single, subeffective dose of a carcinogenic hydrocarbon was followed by repeated applications of croton oil (*1–3*). In such studies many skin tumors occurred rapidly; the quality of the tumors induced, however, differed from those induced by repeated applications of carcinogenic hydrocarbons, in that many of the tumors regressed and few eventually became malignant (*4*). Setälä and his colleagues (*5–8*) have reported that polyoxyethylene sorbitan monostearate (Tween 60) can act as a promoting agent in the skin of the mouse. In studies by this group, mice treated with single doses of carcinogenic hydrocarbon were subsequently given repeated large doses of Tween 60 and developed many benign tumors and a few carcinomas. The experiments with Tween 60 differed from those with croton oil chiefly in the amount of material necessary to induce tumors. Merenmies (*8*) observed that, whereas daily applications of Tween 60 were effective as a promoting stimulus, twice weekly applications of this material were without activity. Setälä and

[1] Received for publication February 23, 1960.

[2] Supported by grant CS-9212, National Cancer Institute, National Institutes of Health, Public Health Service.

[3] A preliminary report of this investigation was presented at the 50th annual meeting of the American Association for Cancer Research, Atlantic City, N.J., April 10 to 12, 1959.

[4] The authors acknowledge the technical assistance of Miss Kay Spencer and Mrs. Birute Zidonis.

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his group (*5–8*) studied several other nonionic, surface active agents and suggested that this type of substance acts through a physicochemical mechanism (*9*).

In a preliminary report (*10*) we noted that Tween 60 was similar to croton oil in that many of the induced tumors regressed. Setälä (*6*) had originally found that Tween 60 alone gave rise to a certain number of skin tumors; more obvious carcinogenic action with Tween 60 alone was noted in our studies. It was additionally demonstrated that, although fewer tumors were induced when Tween 60 was applied twice weekly as a promoting agent or carcinogen, it was nevertheless effective. In the present experiment, the effects of Tween 60 as a promoting agent have been investigated under a variety of conditions: (1) A comparison has been made between different quantities applied during each application, the frequency of treatment being the same; (2) further studies with different frequencies of application have been made; (3) different carcinogenic hydrocarbons have been used as initiating agents; (4) various intervals have been allowed to elapse between the initial application of the carcinogenic hydrocarbon and Tween 60 treatment; and (5) the effect of caging mice individually as compared with 5 to 10 in a cage has been studied. Throughout these experiments, a detailed weekly record has been kept of all tumors induced and their complex life history is discussed.

MATERIALS AND METHODS

Swiss albino mice, originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and from 1951 bred randomly in this laboratory, were used in these studies. The mice were housed in plastic cages with wood shavings, fed Rockland mouse diet in pellets, and received tap water *ad libitum*. The animals were 10 weeks old at the beginning of the experiments, were individually numbered by ear clipping, and weighed; only females weighing from 20 to 26 gm. and males weighing from 24 to 30 gm. were used. The females were kept in groups of 10 per cage and the males in groups of 5 to 10, with the exception of one study in which the mice were caged individually. An area of skin 1.5 × 2 cm. in the interscapular region was clipped free of hair with electric clippers 1 week before treatment; only mice which did not have a gross regrowth of hair were then used. Subsequently the treated area was kept free of hair by clipping with fine scissors.

The carcinogenic hydrocarbons used were 7,12-dimethylbenz[*a*]anthracene (DMBA), 3-methylcholanthrene (MCA), Eastman Organic Chemicals, and benzo[*a*]pyrene (BP), Hoffman-LaRoche, Inc.; the DMBA was purified by chromatography on Florisil. Mineral oil (Superla 34, Standard Oil of Indiana) and reagent grade, redistilled acetone were used as solvents. DMBA in mineral oil was applied as a 1 percent solution. A glass dropper was used to deliver a 25 μl. drop containing 250 μg. Acetone solutions for the various experiments with DMBA, MCA, or BP were at concentra-

tions of 0.04, 0.1, or 0.2 percent and were applied evenly to the shaved skin with a 50 μl. calibrated micropipette. These carcinogens were applied only as single doses. Polyoxyethylene sorbitan monostearate (Tween 60), kindly provided by Atlas Powder Co., Wilmington, Delaware, was applied undiluted at 36° C. from a standard glass dropper delivering drops of approximately 30 mg. It was applied either twice or 5 to 6 times weekly, according to the experiments, for 50 to 80 weeks. For brevity, the 5 to 6 times weekly treatment will hereafter be referred to as daily treatment.

The mice were carefully checked each week, and all tumors occurring in the treated area or elsewhere were charted on graph paper, particular attention being paid to location, size, and shape of the skin tumors. Because of the multiplicity of tumors and the rapidity of their growth and regression, it was occasionally impossible to tell whether a tumor appearing was a new lesion or merely a tumor that had previously regressed. All such lesions have been classified arbitrarily as new tumors. All tumors present for over 1 week have been recorded in the present tabulation. Only sharply defined, raised lesions, larger than 1 mm., were included as tumors. The weight of the mice was recorded every 2 weeks on a cage basis, and no general toxic effect even with the largest doses of Tween 60 was noted. Local skin damage was, however, obvious in the mice treated on a daily basis and consisted of some reddening, thickening, and scaling. The mice were kept until they died, or they were killed if in poor condition. A complete autopsy was performed on every animal, with the exception of a few lost through cannibalism. Histological examination of the skin was invariably undertaken, and, additionally, of all organs exhibiting gross changes. The morphology of the skin tumors is described in another paper.

RESULTS

Tween 60, 1 Drop Twice Weekly or Daily

Three groups of 30 female mice were given 1 drop (30 mg.) of Tween 60 only, twice weekly or daily, or 1 dose of 1 percent DMBA in mineral oil, followed 1 week later by Tween 60 as 1 drop twice weekly. As reported in table 1, only the group prepared with DMBA developed an appreciable number of tumors, which for the most part regressed. The only malignancy observed was a skin fibrosarcoma. Thus no carcinogenic action could be detected when Tween 60 was administered at a level of 30 mg. per dose, whether this was given twice weekly or daily. On the other hand, this threshold was overcome by an initial application of DMBA, and promoting activity was observed.

Tween 60, 2 Drops Twice Weekly With or Without Prior Single Dose of DMBA

Three groups of 30 female and 30 male mice each were treated twice weekly with 2 drops (60 mg.) of Tween 60 for 75 to 80 weeks. One week

TABLE 1.—Tween 60, 1 drop twice weekly or daily

Treatment*	Number of mice	Survivors (*weeks*) †				Number of tumor-bearing animals	Total No. of skin tumors	Number of regressions	Number of skin carcinomas
		20	30	40	50				
DMBA, 1 percent in mineral oil, once, followed by Tween 60, 1 drop twice weekly	30♀	30	28	26	24	13	39	27	0‡
Tween 60, 1 drop twice weekly	30♀	30	30	28	22	2	2§	0	1‖
Tween 60, 1 drop daily	30♀	30	23	21	15	1	1	0	1‖

*Treatment with Tween 60 lasted for 50 weeks.
†From the beginning of Tween 60 treatment.
‡One fibrosarcoma was observed in the treated area.
§One sebaceous adenoma on the face.
‖Squamous-cell carcinoma at perineal site.

before Tween 60 treatment 2 groups were given a single application of DMBA, either in mineral oil or in acetone. In table 2 the results are presented, divided into periods, but not in a cumulative fashion. In each column the number of new tumor-bearing animals, new tumors, and new regressions occurring in each period are represented. For each period the number of animals dying and their tumors are given; the number of animals dying with no tumors are in parentheses. Tween 60 administered twice weekly alone, in individual doses of 60 mg., manifested clear evidence of carcinogenic action. It is of interest to note that the total dose received by these mice was less than that received by the animals of the previous group, which were given 30 mg. daily and developed no tumors. It appears that the quantity of the individual dose is of great importance. When a single dose of DMBA, in mineral oil or in acetone, was applied previously, more tumors, either benign or malignant, arose considerably earlier than in the groups given Tween 60 alone.

Tween 60, 2 Drops Daily With or Without Prior Single Dose of DMBA

Three groups of mice, divided as in the previous experiment, were given Tween 60, as 2 drops (60 mg.) per application, daily for 60 to 82 weeks. The results are presented in table 3, with the use of the same criteria as in table 2. It can be seen that the daily application of Tween 60 alone at this dose level induced a large number of tumors, including several carcinomas, in many animals. The activity with this quantity of material was considerably greater than with twice weekly applications at this dose level. When this quantity of Tween 60 was preceded by a single application of DMBA in either mineral oil or acetone, again the tumors appeared considerably earlier. In terms of the final total number of tumors, however, only the males initially treated with DMBA developed significantly more tumors than animals given Tween 60 only, though in both sexes a greater number of squamous-cell carcinomas were seen in the initiated animals.

Mice Caged Individually and Treated With Tween 60, 2 Drops Daily, After Single Dose of DMBA

Thirty females and 30 males kept 1 to a cage were given a single dose of 20 μg. of DMBA in acetone and, after an interval of 1 week, Tween 60, 2 drops daily. The Tween 60 treatment lasted for 50 weeks; the survivors were kept under observation for an additional period of 10 weeks and then killed. In both groups the survival rate was poor, only 12 females and 12 males surviving at the end of the 40th week of treatment. No obvious reason for this early death was discovered. The results are presented in table 4, which was prepared in the same way as table 2. This experiment was undertaken primarily to determine the effect of caging mice individually on the regression rate of tumors. The results obtained were comparable to those of the other experiments, the regression rate being the same. In the various studies reported in this series, it has

TABLE 2.—Life history of tumors after treatment with Tween 60 given as 2 drops twice weekly, with or without prior single dose of DMBA

Treatment*	Number of mice	†	Variations during time indicated (*weeks*)						Total	Number of squamous-cell carcinomas
			Up to 20	21–30	31–40	41–50	51–60	Over 60		
DMBA, 1 percent in mineral oil, once, followed by Tween 60, 2 drops twice weekly	30 ♀	TBA	6	2	4	7	0	1	20	
		Tumors	11	7	7	33	11	12	81	6‡
		Regr.	4	5	3	19	11	9	51	
		D/T	(1)	(5)	(3) 1/3	(4) 3/7	2/7	(3) 8/13	(16) 14/30	
	30 ♂	TBA	17	1	2	1	2	0	23	
		Tumors	70	4	12	8	12	6	112	4§
		Regr.	33	17	11	8	4	6	79	
		D/T	(1) 2/9	(3) 1/1	(3) 2/5	(6) 2/5	(2) 4/6	(2) 2/7	(17) 13/33	
DMBA, 20 μg. in acetone, once, followed by Tween 60, 2 drops twice weekly	30 ♀	TBA	5	4	5	2	3	1	20	
		Tumors	11	10	16	22	13	4	76	
		Regr.	3	8	16	10	11	5	53	6‖
		D/T	0	0	0	(2)	(4) 2/3	(12) 10/20	(18) 12/23	
	30 ♂	TBA	9	5	1	2	1	1	19	
		Tumors	25	30	20	9	5	12	101	4‖
		Regr.	17	15	30	4	7	13	86	
		D/T	(3)	(1)	(2) 1/1	(1) 1/2	(5) 2/2	(7) 7/10	(19) 11/15	
Tween 60, 2 drops twice weekly	30 ♀	TBA	0	0	5	3	0	1	9	
		Tumors	0	0	9	20	12	6	47	
		Regr.	0	0	0	12	8	12	32	1¶
		D/T	0	0	(2)	(5) 1/1	(9) 2/5	(7) 4/9	(23) 7/15	
	30 ♂	TBA	1	1	3	3	1	1	10	
		Tumors	1	2	10	9	15	7	44	0
		Regr.	0	1	5	10	11	5	32	
		D/T	(5)	(8)	(3)	1/1	(2) 2/2	(7) 2/9	(25) 5/12	

*Treatment with Tween 60 lasted 75 to 80 weeks.

†TBA = new tumor-bearing animals; tumors = new tumors appearing in each period; regr. = tumors regressing in each period. D/T = dying animals/tumors present at death; in parentheses the number of animals dying with no tumors.

‡In addition, 3 basal-cell carcinomas and 1 fibrosarcoma of the skin.

§In addition, 1 malignant tumor of the skin, not examined histologically.

‖In addition, 1 basal-cell carcinoma.

¶In addition, 1 fibrosarcoma of the skin.

TABLE 3.—Life history of tumors after treatment with Tween 60 given as 2 drops daily, with or without prior single dose of DMBA

Treatment*	Number of mice	†	Variations during time indicated (*weeks*)						Total	Number of squamous-cell carcinomas
			Up to 20	21–30	31–40	41–50	51–60	Over 60		
DMBA, 1 percent in mineral oil, once, followed by Tween 60, 2 drops daily	30 ♀	TBA	9	1	3	8	2	0	23	
		Tumors	22	31	30	33	33	14	163	8
		Regr.	7	14	21	40	22	16	120	
		D/T	(1)	0	(4)3/9	(2)	(2)5/14	(3)10/20	(12)18/43	
	30 ♂	TBA	22	0	1	1	0	0	24	
		Tumors	166	19	23	66	14	1	289	
		Regr.	121	40	20	26	24	0	231	8
		D/T	(2)2/3	(3)2/3	(5)5/6	(1)3/19	(3)3/23	1/4	(14)16/58	
DMBA, 20 μg. in acetone, once, followed by Tween 60, 2 drops daily	30 ♀	TBA	17	1	2	1	1	0	22	
		Tumors	56	14	54	14	11	10	159	
		Regr.	31	19	36	24	1	7	118	9‡
		D/T	(3)1/4	0	(2)4/14	3/6	(7)2/2	(3)5/15	(15)15/41	
	30 ♂	TBA	19	3	3	1	0	1	27	
		Tumors	90	35	130	56	45	9	365	11
		Regr.	63	17	63	64	29	5	241	
		D/T	(2)	(2)	(2)6/39	(2)6/24	6/21	4/40	(8)22/124	
Tween 60, 2 drops daily	30 ♀	TBA	2	2	4	7	1	0	16	
		Tumors	3	3	25	71	31	17	150	4§
		Regr.	1	2	6	55	30	20	114	
		D/T	(1)	(3)	(3)1/1	(5)3/4	(2)2/17	(4)6/14	(18)12/36	
	30 ♂	TBA	1	1	1	3	0	—	6	
		Tumors	1	1	5	40	5	—	52	1
		Regr.	0	1	2	9	7	—	19	
		D/T	(3)	(10)	(10)	(1)1/1	(1)4/32	—	(25)5/33	

*Treatment with Tween 60 lasted 60 to 82 weeks, according to the survival rate of each experiment.

†*See* † footnote in table 2.

‡In addition, 1 fibrosarcoma and 1 malignant tumor of the skin not examined histologically.

§In addition, 1 basal-cell carcinoma.

TABLE 4.—Life history of tumors in mice caged individually and given 2 drops of Tween 60 daily after single dose of DMBA

Treatment *	Number of mice	†	Variations during time indicated (*weeks*)					Total	Number of squamous-cell carcinomas
			Up to 20	21–30	31–40	41–50	51–60		
DMBA, 20 μg. in acetone, once, followed by Tween 60, 2 drops daily	30♀	TBA	12	1	0	1	0	14	
		Tumors	45	6	4	7	3	65	2
		Regr.	33	7	9	1	6	56	
		D/T	(8)	(3)	(5)1/1	(1)2/2	(7)3/6	(24)6/9	
	30♂	TBA	21	0	0	1	0	22	
		Tumors	114	5	6	6	1	132	2
		Regr.	100	9	7	2	2	120	
		D/T	(6)2/4	(5)	(5)	(4)2/3	(2)4/5	(22)8/12	

* Treatment with Tween 60 lasted for 50 weeks.
†*See* † footnote in table 2.

been found that the male mice developed more tumors than the females which were treated similarly. This might have been partly attributed to aggressiveness and fighting by the males, which produced skin injury. However, males caged individually still developed more tumors than the females, and clearly some other factor must be involved.

Tween 60 Daily, 15 or 25 Weeks After Single Dose of DMBA

Fifty females and 55 males, 10 weeks old, were given 20 μg. of DMBA in acetone once. After an interval of 15 weeks, treatment with 2 drops daily of Tween 60 was started in 25 females and 25 males. Ten weeks later, that is, 25 weeks after the initial treatment with DMBA, the surviving 20 females and 20 males were treated with Tween 60. Another group of 50 females and 52 males of the same age were kept with no hair clipping and no treatment; of these, 25 females and 25 males were treated with Tween 60 15 weeks later, and 23 females and 18 males after 10 additional weeks, that is, at 25 and 35 weeks of age, respectively. The number of survivors, the tumor incidence, and the average latent period are reported in table 5. In all groups, the treatment with Tween 60 lasted until the 80th week of age. It can be seen that, when an interval of 15 weeks was allowed to elapse between the DMBA and Tween 60 treatment, the results were essentially similar to those obtained with an interval of only 1 week (table 3). The tumors were more numerous than those obtained with Tween 60 alone and occurred earlier. When there was an interval of 25 weeks between the two treatments, little difference could be seen between these mice and those treated with Tween 60 only. The controls with Tween 60 only were 15 and 25 weeks older than the mice usually used. It is interesting that in the latter mice the few tumors induced appeared sooner than in any other groups given Tween 60 alone.

Tween 60, 2 Drops Daily After Single Dose of DMBA, BP, or MCA

Four groups of 20 females and 20 males each were given a single dose of either 50 or 100 μg. of DMBA, 50 μg. of BP, or 50 μg. of MCA. One week later, all the groups were treated daily with 2 drops of Tween 60 for 50 weeks. The results are reported in table 6. In the 2 groups treated with different doses of DMBA, clear initiating action, comparable to that in other groups, occurred; there was, however, little effect correlated with different dosage levels. The groups treated with BP and MCA developed fewer tumors than the groups given DMBA and, proportionately, even less than mice given Tween 60 alone (table 3). However, in the BP and MCA initiated groups, 60 to 87 percent of the tumors arose before the 30th week of treatment, while only 4 percent of the tumors had occurred at this time in the groups treated only with Tween 60.

Control Groups Given Single Application of Carcinogenic Hydrocarbons Only

As recorded in table 7, 5 groups of mice were given a single dose of either 20, 50, or 100 μg. of DMBA, 50 μg. of BP, or 50 μg. of MCA dis-

TABLE 5.—Tween 60, 2 drops daily, starting 15 or 25 weeks after single dose of DMBA

Treatment*	Number of mice	Survivors (*weeks*)†						Number of tumor-bearing animals	Total No. of skin tumors	Number of regressing tumors	Number of squamous-cell carcinomas	Average latent period‡
		15	25	30	40	50	60					
DMBA, 20 μg. in acetone, once, followed 15 weeks later by Tween 60, 2 drops daily	28♀	25	25	25	22	18	7	16	96	80	1	15. 29
	30♂	25	23	21	15	6	5	19	93	79	0	11. 93
DMBA, 20 μg. in acetone, once, followed 25 weeks later by Tween 60, 2 drops daily	22♀		20	20	17	15	4	9	37	25	1	15. 81
	25♂		20	19	12	6	1	7	17	10	0	9. 64
Tween 60, 2 drops daily, starting at 25 weeks of age	25♀	25	23	23	23	18	9	9	11	8	0	27. 09
	29♂	25	25	23	16	9	0	3	6	3	0	20. 50
Tween 60, 2 drops daily, starting at 35 weeks of age	25♀		23	23	22	16	10	10	18	13	0	18. 44
	23♂		18	17	14	9	4	4	12	7	0	15. 33

*DMBA treatment given at 10 weeks of age.

†From time of 10 weeks of age.

‡Calculation from the beginning of Tween 60 treatment, for the total number of skin tumors.

TABLE 6.—Tween 60, 2 drops daily after single dose of DMBA, BP, or MCA

Treatment*	Number of mice	Survivors (*weeks*)					Number of tumor-bearing animals	Total No. of skin tumors	Number of regressing tumors	Number of squamous-cell carcinomas
		20	30	40	50	60				
DMBA, 50 μg. in acetone, once, Tween 60, daily	20 ♀	20	16	9	7	5	15	72	58	1
	20 ♂	17	14	3	3	1	14	113	85	1†
DMBA, 100 μg. in acetone, once, Tween 60, daily	20 ♀	20	15	10	9	7	16	110	81	4‡
	20 ♂	20	19	10	8	4	18	113	106	0§
BP, 50 μg. in acetone, once, Tween 60, daily	20 ♀	20	20	16	11	10	11	33	25	2
	20 ♂	18	12	7	3	3	10	30	24	0
MCA, 50 μg. in acetone, once, Tween 60, daily	20 ♀	19	19	13	10	8	11	30	25	0‖
	20 ♂	19	17	6	3	2	14	32	30	0

*Treatment with Tween 60 consisted of 2 drops per application and lasted for 50 weeks.

† In addition, 2 basal-cell carcinomas.

‡ In addition, 1 malignant tumor of the skin not examined histologically, 1 basal-cell carcinoma, and 2 fibrosarcomas of the skin.

§ One fibrosarcoma of the skin.

‖ In addition, 1 basal-cell carcinoma.

TABLE 7.—Control groups given single application of carcinogenic hydrocarbons only

Treatment*	Number of mice	Survivors (*weeks*) †								Number of tumor-bearing animals	Total No. of skin tumors
		20	30	40	50	60	70	80	90		
DMBA, 20 μg., once	60 ♀	57	54	49	48	34	27	7	0	2	2‡
	32 ♂	30	27	22	17	10	5	0	0	2	2§
DMBA, 50 μg., once	22 ♀	22	22	22	21	20	18	12	8	2	2‖
DMBA, 100 μg., once	32 ♀	29	29	28	24	19	16	7	3	3	3¶
BP, 50 μg., once	22 ♀	22	21	21	19	16	7	4	1	0	0
MCA, 50 μg., once	22 ♀	22	22	22	18	15	11	7	6	1	2**

*The hydrocarbons were dissolved in acetone and given as a single application in 50 μl.
† From time of treatment.
‡ One squamous-cell carcinoma on ear; 1 papilloma on eyelid.
§ One fibrosarcoma on foreleg; 1 papilloma on treated area.
‖ One basal-cell carcinoma of ear duct; 1 acanthoma on abdomen.
¶ One basal-cell carcinoma of ear duct; 2 papillomas on ear, one regressing.
**One squamous-cell carcinoma of ear duct; 1 acanthoma on treated area.

solved in 50 μl. of acetone. In the groups given 20 μg. of DMBA, most of the survivors were killed between the 70th and the 80th week of the experiment; in the other groups the survivors were killed after the 90th week. As is shown in table 7, very few skin tumors were observed: 11 in all in 190 mice, only 2 located in the treated area. The first tumor appeared at the 43d week; the average latent period for the 11 tumors was 65.8 weeks.

Skin Tumors Outside Treated Area

Approximately 10 percent of all skin tumors, observed in the experiments with Tween 60, were located outside the treated area, mainly on the ears, occasionally on the face, perineal region, or abdomen. These tumors, with few exceptions, appeared late, with an average latent period of 47 weeks. No appreciable difference was noted between the mice receiving the initial treatment with hydrocarbons and those given Tween 60 only. Most of the tumors appearing in the control groups, given a single dose of hydrocarbons only, were outside the treated area, as in the experiments reported by Roe (*11*).

Pathological Findings

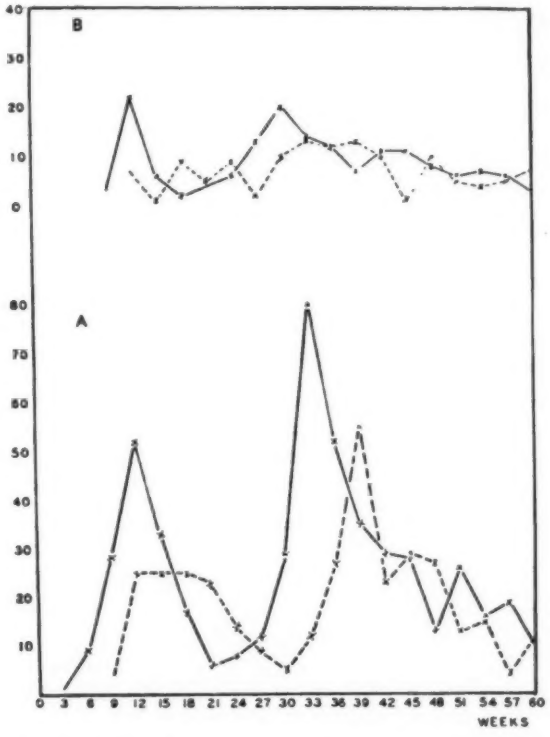
In almost all animals careful autopsy and histological study were performed. A morphological study of the skin tumors is reported in another paper. In the animals given the continuous treatment with Tween 60, hyperplasia of the epidermis and inflammatory changes of the treated skin were always present, though with varying intensity. Chronic pyelonephritis and hyaline changes in kidneys, liver, spleen, and adrenals were often seen. The incidence of malignant lymphomas, granulocytic leukemias, lung adenomas, and mammary adenocarcinomas did not differ from that seen in our untreated Swiss mice.

DISCUSSION

Four different experiments with the use of Tween 60 alone are recorded. In the first 2 experiments (table 1) female mice received 1 drop (approximately 30 mg.) of Tween 60 either twice weekly or daily. In neither case were any tumors attributable to the treatment observed. In the second 2 experiments, mice of both sexes were given 2 drops (60 mg.) of Tween 60 either twice weekly (table 2) or daily (table 3). In both instances considerable numbers of tumors were induced, more when the treatment was given daily than only twice weekly. A total of 5 squamous-cell carcinomas were observed in the group given 2 drops of Tween 60 daily and only 1 in the group treated twice weekly. From 93 to 100 percent of these tumors arose after the 30th week of treatment. It is thus established that Tween 60 has a carcinogenic action on the skin of mice, albeit a weak one, and that there is a marked dosage response with an apparent threshold. The

quantities of material needed to induce tumors are literally enormous, absolutely, and by comparison with known carcinogens.

When the Tween 60 treatment is preceded by a single initiating dose of a carcinogenic hydrocarbon, a similar dose response is seen. Under these conditions, even the twice weekly applications of a single drop of Tween 60 gave rise to tumors (table 1), though it can be seen from table 2 and 3 that twice weekly and daily applications of 2 drops of Tween 60 after a single dose of DMBA are correspondingly more effective. This finding confirms the results of Merenmies (*8*), who found a similar dose-



TEXT-FIGURE 1.—Incidence of appearance and regression of tumors in experiments with DMBA initiation and Tween 60 promotion.

A: Thirty females and 30 males given DMBA, 20 μg., once, followed by Tween 60 daily (*see* table 3).
B: Thirty females and 30 males given DMBA, 20 μg., once, followed by Tween 60 twice weekly (*see* table 2).

Each *cross* on the *continuous* line represents the number of tumors appearing during the 3 preceding weeks. Each *cross* on the *dotted* line represents the number of tumors regressing during the 3 preceding weeks. Tumors and regressions of females and males were added together.

response relationship, using different concentrations of aqueous solutions of Tween 60 and grading the weekly frequencies of the applications. On the other hand, Graffi (*12*), using 1 or 2 weekly applications of croton oil as promoter, only found a difference in latent period.

In a comparison of the results of the experiments with Tween 60 alone and those preceded by a single application of DMBA (tables 2 and 3), there are obviously considerable differences in the number of tumors seen and in the latent periods. The most obvious difference is the tumors seen early up to the 30th week in the studies preceded with DMBA. In most of the groups, there was a second growth of tumors after the 30th week of treatment with Tween 60, which resulted in a biphasic pattern (text-fig. 1). This second peak of tumors approximately paralleled in time the occurrence of tumors in the experiments in which Tween 60 was given alone. During the first 30 weeks, the male mice consistently developed more tumors, ranging from 178 to 349 percent of the tumors appearing in the females in the same period. During the second part of the experiment, the response of female and male mice was similar in almost all groups, though complicated by different survival rate. The number of squamous-cell carcinomas did not differ significantly in the 2 sexes. In experiments with croton oil, Salaman and Roe (*13*) reported a similar sex difference, males bearing almost twice as many papillomas as females, while the incidence of malignant tumors was almost the same in the 2 sexes.

The dose of carcinogen used as initiator, when given alone as a single application, either in acetone (table 7) or in mineral oil (*14*), gave rise to almost no tumors. It is therefore difficult to imagine a simple additive effect that could account for the action of the two agents, DMBA and Tween 60, given in sequence. In particular, the time distribution of the tumors appears to indicate a promoting effect.

In studies with DMBA followed by croton oil (*15*, *16*), intervals up to 43 weeks may be left between the initiating dose and the beginning of the promoting treatment without an alteration in the level of tumors induced. In the present studies, groups of mice have been treated with 20 μg. of DMBA and subsequently with Tween 60 daily, after intervals of 15 to 25 weeks (table 5). After the interval of 15 weeks, considerably more tumors were induced than could be accounted for by the Tween 60 alone; additionally, the average latent period was significantly shorter in the group receiving the initiating dose of DMBA. In the experiments with an interval of 25 weeks, however, no differences were observed. It is difficult to attach great significance to this finding due to the small number of survivors at this late age.

With croton oil as the promoting agent, varying the dosage of initiating agent (DMBA) resulted in the induction of a correspondingly different number of tumors (*17*, *18*). In the present studies, when animals treated similarly with Tween 60 were initially exposed to 20, 50, or 100 μg. of DMBA in acetone, no marked differences in tumor incidence that could

be related to the DMBA dosage were noted (tables 3 and 6). More studies are needed to clarify this situation. The effect of solvent is an important factor, since in our studies 250 μg. in mineral oil gave a yield of tumors similar to that seen with 20 μg. of DMBA in acetone (tables 2 and 3); it seems possible that the lowest dose in these studies may have corresponded to the higher doses in the previous studies (*17*), and that investigations in other dose ranges may be needed to reveal a dose response. In the other 2 studies recorded in table 6, 50 μg. of BP or MCA was the initiating agent. In neither of these studies were more tumors induced by applications of Tween 60 than were induced by this agent alone, but the majority of tumors occurred during the first 30 weeks of treatment, as in the groups initiated with DMBA.

The most striking phenomenon throughout all experiments with Tween 60 alone, or after a single dose of carcinogenic hydrocarbon, was the behavior of the lesions. As can be seen from the tables, the majority of tumors regressed before the end of the study. Of a total of 2,701 tumors appearing in all these studies, 2,040 regressed, only 24.5 percent being present at the death of the animals. The distribution in time of the occurrence and regression of the tumors seen in 2 representative experiments is shown in graphic form in text-figure 1. It is apparent that the regressions followed the pattern of the tumor inception closely, the majority occurring within a few weeks of the appearance of tumors. No significant differences in the percentage of the regressing tumors were seen with these different experimental conditions. On the whole, the rate of regression of tumors observed in the present studies seems to be similar, though higher, than those observed in experiments with croton oil after initiation (*4*, *14*) and in experiments with a single large dose of carcinogenic hydrocarbons (*19*, *21*). However, in the study of Mider and Morton (*19*) and in unpublished experiments from our laboratory, it appears that the regression of tumors, induced with a single dose of carcinogen, follows the inception of tumors after a longer delay than that observed in the Tween 60 experiments. It should be observed that the percentage of regressing tumors after repeated applications of polycyclic hydrocarbons is much lower (*22*) or nil (*14*).

One possible reason for the high regression rate in our experiments was the presence of 5 or more animals per cage, which might result in the mice eating or scratching off the tumors. To control this possibility one experiment was undertaken in which the mice were housed individually. This led to similar results as can be seen in table 4. Thus it appears that the phenomenon of regression must be related to the nature of the tumors induced. Rous and Kidd (*23*, *24*) found that induced tumors in rabbit skin often regressed when the tarring was suspended, and thus differed from croton oil and Tween 60 experiments in which regressions occurred while the treatment was continuing. It is possible that the continuous irritation produced by the treatment may play a role in the mechanism of these regressions.

A few squamous-cell carcinomas were seen in most of the mice given Tween 60 after a single dose of carcinogenic hydrocarbons, and more in the groups receiving Tween 60 daily than twice weekly (tables 2 and 3). The fewer carcinomas observed in the group of mice individually caged (table 4) or given different initiation (table 6) may be related to poor survival rate and the smaller number of mice. Some of the carcinomas clearly arose from a preceding papilloma-type lesion; for the majority, however, it was difficult to ascertain the pathway and moment of inception. An average latent period for carcinomas is thus difficult to assess, although undoubtedly the majority appeared after the 40th week of the experiment. It must be borne in mind that the numerical relationship of carcinomas to the total quantity of tumors induced is complicated by the number of regressions, the number of tumors present in the dying animals, and the survival rate. In the mice receiving an initiating dose of DMBA 1 week prior to Tween 60, of a total of 1,990 tumors only 66 squamous-cell carcinomas were eventually seen. This incidence of malignant transformation of 3.2 percent is very small. This finding is comparable to that seen in the croton oil experiments (*4*, *13*, *14*) and strikingly different from that in the experiments which used repeated applications of carcinogenic hydrocarbons (*14*, *22*, *25*).

In conclusion, from all the experiments reported, it is obvious that Tween 60 has a promoting action on the skin of mice comparable to that of croton oil. Originally (*4*, *26*, *27*) no tumors or an occasional one was reported after treatment with croton oil alone, though later several investigators found croton oil to induce tumors after a longer period of treatment (*28–30*). Similarly, Setälä (*6*) reported that Tween 60, when applied to mice not previously initiated, produced only occasional tumors. In our experiments a large number of tumors with few malignancies were induced by Tween 60 alone. This observation only partially contradicts the two-stage theory of skin carcinogenesis, if one observes that the latent period with Tween 60 and croton oil is very different, tumors arising rapidly after initiation and much later without initiation. In this respect the biphasic pattern observed in our experiments, which had good survival rates, adds further information; it seems that the second peak of tumors seen after initiation must have similar biological significance to the group of tumors induced by Tween 60 alone.

Like croton oil, Tween 60 is a complex chemical mixture; in croton oil, preliminary fractionation studies revealed that promoting activity may be concentrated into a small moiety of the whole (*27*, *31–33*) and it would, therefore, appear likely that a specific agent will eventually be isolated that possesses this activity. In Tween 60, the dose-response situation observed reveals that the important factor is the quantity applied in the individual application; since the quantity needed is so large, it appears possible that this is a gross physical phenomenon perhaps quite different from the action of other chemicals in skin carcinogenesis. It might be analogous, for example, to the induction of sarcomas by implantation of plastics (*34*).

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Induction of Melanotic Tumors in the Syrian Golden Hamster After Administration of Ethyl Carbamate [1, 2, 3]

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SUMMARY

Ethyl carbamate (urethan), when given as 0.2 percent solution in drinking water to female and male Syrian golden hamsters, induced in the males a high incidence of melanotic tumors similar to those elicited in this species by 7,12-dimethylbenz[*a*]anthracene (DMBA). In both sexes a high incidence of papilloma of the forestomach was also observed. Cutaneous applications of 20 percent urethan failed to show a tumorigenic effect.—J. Nat. Cancer Inst. 25: 627–630, 1960.

MELANOTIC TUMORS similar to the cellular blue nevus of man have been induced in the Syrian golden hamster by cutaneous application of 7,12-dimethylbenz[*a*]anthracene (*1–3*). The induction of these tumors by a single dose as low as 0.05 mg. has been reported (*4*). Related polycyclic hydrocarbons, such as 3-methylcholanthrene, benzo[*a*]pyrene, dibenz[*a*,*h*]anthracene, benz[*a*]anthracene, and the compound 2-anthramine, when applied under similar experimental conditions, failed to elicit melanotic tumors (*4*).

The induction of melanotic tumors in this species after administration of ethyl carbamate (urethan) is reported in the present study.

MATERIAL AND METHODS

Two groups of 8- to 10-week-old Syrian golden hamsters, obtained from Abrams Small Stock Breeders, Chicago, were used. The animals were housed in plastic cages on wood shavings in groups of 5 per cage, according to sex and fed a Rockland rat diet in pellets, with water *ad libitum*. The first group composed of 10 females and 10 males received a 0.2 percent solution of urethan in their drinking water. Twice weekly,

[1] Received February 28, 1960.

[2] This investigation was supported by grant C-2893 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] A preliminary report was presented at the Seventh International Cancer Conference, London, July 1958.

[4] The authors wish to acknowledge the technical assistance of Mrs. Shirley Freundlich.

cutaneous applications of 20 percent urethan dissolved in redistilled acetone were given to a second group of 10 females and 10 males. Each application consisted of 6 drops evenly distributed on the skin of the middle back on an area, approximately 6 × 3 cm., kept free of hair with an electric clipper. The animals were identified by ear clipping, checked at weekly intervals, and changes on the skin were recorded on graph paper. Those pigmented lesions having a nodular appearance and reaching at least 2 mm. in size were classified as melanotic tumors; other pigmented and nonpigmented cutaneous lesions were also carefully recorded.

Urethan was administered for the normal lifespan of the animals. The average daily consumption of water per animal was 10 ml., which corresponded to 20 mg. of urethan. The cutaneous application consisted of 30 mg. of urethan.

Necropsies were performed on all dead animals with the exception of one animal lost through cannibalism Skin and other organs showing gross pathological changes were examined microscopically.

RESULTS

It is evident from table 1 that melanotic tumors were induced after oral administration of urethan but that skin application was without effect. A marked sex difference was observed. Only one unquestionable melanotic tumor was recorded at the 30th week of the experiment in the female group given urethan by mouth. In contrast to this, melanotic tumors occurred in 7 of the 10 males, the first appearing at the 29th week and the last at the 66th week, with a mean latent period of 56 weeks. Although the females did not develop more tumors, 20 melanotic spots, not large enough to be classified as tumors, were observed. In the male hamsters, in addition to the tumors, 65 melanotic spots were observed in 8 animals. The marked sex difference in the response to the oral treatment with urethan is difficult to evaluate because of the shorter lifespan of the female animals. The majority of these died within the 1st year of the experiment, thus receiving less of the carcinogen and having less opportunity to develop macroscopically visible tumors. The melanotic spots and tumors showed a predilection for the more heavily pigmented skin of the back and forehead; however none was seen on the most pigmented areas, namely, the tips of the ears, the dorsolateral scent glands, and the anal region. A single melanotic tumor and a few pigmented spots were found on the abdominal skin.

These melanotic tumors are identical histologically with those seen after treatment with DMBA *(1)*. Negative results were obtained with skin application of urethan and they may well be due to lower dosage.

In addition to the melanotic tumors, a highly significant incidence of papillomas of the forestomach was observed both with oral and skin treatment. The forestomach of the mouse has already been reported to

TABLE 1.—Effects of administration of ethyl carbamate to Syrian golden hamsters

Treatment	Number of animals	Survivors at weeks: 10	20	30	40	50	60	70	Last animal died (*in weeks*)	Number of animals with melanotic tumors	Number of melanotic tumors: 2–4 mm.	5–9 mm.	10 mm.	Other neoplasms
Urethan, 0.2% in the drinking water	10♀	10	10	9	7	3	0	0	55	1	1	0	0	26*
	10♂	10	10	10	9	9	6	6	76	7	16	4	1	32†
Urethan, 20% to the skin, 6 drops twice weekly	10♀	10	10	10	8	7	1	0	68	0	0	0	0	6‡
	10♂	9	9	9	9	9	8	5	3 alive at 95 weeks	0	0	0	0	0

*Four animals with 24 papillomas of the forestomach and 1 adenoma of thyroid; 1 animal with a metastasizing hemangiosarcoma of liver.

† Six animals with 30 papillomas of the forestomach and 1 malignant lymphoma; 1 with a bronchial adenoma carcinoid type.

‡ Two animals with 6 papillomas of the forestomach.

be highly sensitive to the action of urethan (*5*); the hamster appears to have the same characteristics.

As a control, 14 female and 49 male Syrian golden hamsters have been kept untreated for the past 80 weeks. A single melanotic tumor measuring 2 × 2 mm. was recorded in a male animal that died at the 73d week of age. Papillomas of the forestomach have not been observed in this control group.

DISCUSSION

During several studies on carcinogenesis with the Syrian golden hamster, melanotic tumors have only been induced with DMBA. This appeared to be a singularly specific action since several closely related polycyclic hydrocarbon carcinogens did not manifest this action. The compound 2-anthramine, although inducing a few melanotic spots, did not give rise to these tumors, and the compound *N*-2-fluorenylacetamide, perhaps the most versatile carcinogen, only gives rise to cholangiomas in this species (*6*). It is interesting that of all these compounds tested, only urethan, which does not appear to resemble DMBA in most other respects, should elicit the same neoplastic response. The effects of urethan, once thought only to give rise to lung adenomas in mice, have resulted in the induction of a variety of neoplastic lesions. Salaman and Graffi (*7*, *8*) showed that urethan was capable of initiating skin carcinogenesis; Tannenbaum reported (*9*) that it produced mammary tumors, cystadenomas of the lacrimal glands, and malignant mesenchymal tumors of the interscapular fat pad in mice. This report adds yet another tumorigenic effect to this list.

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Studies on the Mechanism of Immunological Enhancement of Tumor Grafts [1]

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SUMMARY

The mechanism of immunological enhancement of tumor homografts by heteroimmune serum was studied, particularly the immunological properties of the active antiserum and its mode of action. Tumor homografts, enhanced by heteroimmune serum, were shown to elicit an intense homograft reaction, while growing progressively in foreign hosts. This reaction is manifested both by the production of isohemagglutinins with enhanced 6C3HED lymphosarcoma and by the increased resistance of the host, *i.e.*, transplantation immunity produced by enhanced Sarcoma SBL1. The enhancement of tumor homografts was thus attributed to a capacity to resist transplantation immunity induced in the tumor cells and not to suppression of the host's resistance. This implies a direct action of the enhancing antibodies on the tumor cells. The direct action was further supported by the fact that heteroimmune serum enhanced the growth of tumor isografts. Passive transfer of immunity through lymph nodes activated by tumor homografts of Sarcoma MC1M revealed that after a prolonged exposure the same lymph nodes producing transplantation immunity can produce enhancing antibodies. The experimental data obtained in the present study might explain immunological enhancement if it is assumed that the enhancing antibodies stimulate an augmented synthesis of their homologous antigen in the tumor cells. Such an increased production of antigen might explain both the augmented growth of enhanced tumor isografts and the resistance of enhanced tumor homografts to transplantation immunity.—J. Nat. Cancer Inst. 25: 631–648, 1960.

"THE TERM immunological enhancement applies specifically to the successful establishment of a tumor homograft and its progressive growth as a consequence of the tumor's contact with specific antiserum in the host" (*1*). The recent literature on this apparent paradoxical phenomenon has been reviewed thoroughly by Kaliss (*1*), who made a valuable contribution to the analysis of the problem, after the preliminary work of Casey (*2*) and the studies of Snell (*3*). The active factors in the induction of immunological enhancement have been demonstrated as humoral antibodies (*4*); yet the mechanism by which they induce the process has

[1] Received for publication February 24, 1960.
[2] We wish to thank Mr. Z. Kornbaum and Mr. S. Leib for their skillful technical assistance.

until recently remained speculative. A number of hypotheses have been suggested to explain the phenomenon: According to one, the enhancing antibodies cross-react with the tumor's transplantation antigens of the *H-2* system and prevent the latter from evoking the usual host resistance, *i.e.*, transplantation immunity to the tumor homografts (*5*, *6*); a second holds that the active antibodies exert a selective pressure by which cells compatible with the foreign host are selected from the original cell population of the tumor (*7*); the third (*1*, *8*) claims that a "physiological" change of an unidentified type takes place in the tumor cells, which enables the homografts to survive. However, neither the postulated "cross-reaction" nor the properties of the "physiologically changed" tumor have been experimentally demonstrated. The present study has been undertaken to test the suggested theories and to analyze (*a*) the types of antibodies constituting the enhancing antiserum, (*b*) their site of formation in relation to the site of formation of transplantation immunity, (*c*) whether the antibodies act by suppressing the homograft reaction, or (*d*) by a direct action on the tumor cells. In the latter case (*e*) what are the properties conferred on the tumor cells which ensure its progressive growth and (*f*) how do the antibodies induce these properties?

The present communication is a first report on experiments related to these questions, which were based mainly on the effect of heteroimmune serum in the induction of immunological enhancement.

MATERIALS AND METHODS

Animals.—Mice used in the present study were of C3H, C57BL, and Swiss strains, from the same stocks used in previous studies in this laboratory (*9*, *10*). The mice were 3 to 3½ months old at the beginning of the experimental treatment.

Rabbits were of a heterogeneous stock, 5 months old at the beginning of immunization.

Tumors.—The following tumors were used: the ascites forms of 6C3HED, 6C3HED-ICR, MC1M, and MC1A, all of C3H origin. The immunogenetic properties of these tumors have been previously studied (*9*, *10*). The 6C3HED is a strain-specific lymphosarcoma; MC1M is a semihomotransplantable sarcoma that in foreign strains gives successful homografts intraperitoneally but not at other sites. MC1A and 6C3HED-ICR are homotransplantable tumors which give successful homografts both intraperitoneally and intramuscularly.

Sarcoma SBL1 is a strain-specific tumor produced in our laboratory in C57BL mice by a subcutaneous injection of benzo[*a*]pyrene (*11*).

Antiserums for induction of enhancement were produced in rabbits according to the procedure described by Kaliss (*12*). Mouse spleens were homogenized in 0.85 percent saline. The homogenate was centrifuged at 500 × g; the supernatant was used for intravenous injections and the sediment for intraperitoneal injections. Each rabbit was given

6 double injections, at 4 to 5 days apart. Each such injection consisted of 1 intravenous inoculation of the supernatant and 1 intraperitoneal inoculation of the sediment, spaced 4 hours apart. Each double injection (supernatant plus sediment) contained tissue from 1 mouse spleen.

Absorptions, either with erythrocytes or with liver cells, were carried out in inactivated samples. Two absorptions with red blood cells, at 37° C., removed all complete agglutinins. Absorptions of antiserums with liver cells were carried out in antiserums diluted 1:4 in saline. Each sample was absorbed twice, at 37° C. for 1 hour, with an equal volume of tissue homogenate.

Serological tests.—(*a*) Hemagglutinins were tested in 1:2 dilutions of 0.1 ml. antiserums, against equal volumes of 3 percent red blood cells in saline. The tests were read after incubation at 37° C. for 1 hour. (*b*) *Incomplete hemagglutinins* were tested by the method of Gorer and Mikulska (*13*) for the *H-2* agglutinins in isoimmune serums. (*c*) *Cytotoxic antibodies* were tested by Gorer and O'Gorman's method for cytotoxins produced in the homograft reaction (*14*). Usually ascites tumors were used for testing cytotoxins. The reactivity of these tumor cells to cytotoxins of isoimmune serums has been previously reported (*15*). In our present study the cells were similarly suspended in normal mouse serum diluted 1:5 in saline. For 1 hour, 0.1 ml. of cells was incubated with 0.1 ml. of antiserum (1:2), to which 0.08 ml. of guinea-pig complement (dehydrated complement from the Texas Biological Laboratory) was added. Cytotoxicity was determined according to the percentage of cells that take up trypan blue (0.05% in Tyrode) after incubation.

The figures given are the means of 3 to 4 replicates of each reaction (3–4 tubes). Two samples of each replicate tube were counted, each sample of 200 cells.

EXPERIMENTS

Serological Properties of Heteroimmune Serum

Toxicity

While studying the effect of rabbit antiserums on lymphoma cells, Mohos and Kidd (*16*) noticed the toxic effect of such antiserums on mice. Since the present study is concerned with the *in vivo* interactions between antiserums and tumor cells, we tested (*a*) the toxicity of heteroimmune serums in mice, (*b*) the serological factors of the toxicity, and (*c*) means for abolishing the toxic effect.

Swiss mice each received intraperitoneal injections of 1.0 ml. of rabbit anti-C3H-spleen serum. Group 1 received untreated antiserum, group 2 inactivated antiserum heated at 56° C. for 1 hour, and group 3 inactivated antiserum heated at 65° C. for 1 hour. Heating procedures were adopted after it was claimed that this abolished the toxicity of rabbit antiserum (*17*). Yet all the animals from all 3 groups died within a few hours after serum injection, whereas normal serum did not cause

any mortality under similar conditions. The induced antibodies are thus responsible for the lethal effect. Similar tests were then made with inactivated antiserum, which was injected after it was diluted 1:4 and 1:5. The animals survived one injection, but when they were given a second injection of diluted antiserum 24 hours later, they died within a few hours. Since Kaliss used lyophilized antiserum (*17*), we similarly lyophilized samples of antiserums. However, the injection of such antiserums was no less toxic than fresh antiserum.

Mice injected with heteroimmune serum showed symptoms of hemoglobinuria; the peritoneum contained agglutinated red blood cells, the spleen was enlarged and dark, and the liver cirrhotic. Blood taken at various time intervals after injection (tail bleedings) showed a gradual agglutination of the erythrocytes.

Serological Tests

Agglutinins

When we found that *in vivo* hemagglutination occurred in the animals receiving injections, tests were set to determine the titers of agglutinins and to check their strain specificity. The results (table 1) showed that the anti-C3H serum agglutinated erythrocytes of both C3H, C57BL, and Swiss mice, showing a titer range of 2^{7-9}. To test whether the removal of these agglutinins would eliminate the toxic effect of the antiserums, the latter were inactivated, absorbed with Swiss erythrocytes, and injected intraperitoneally into mice. The absorbed antiserums did, however, retain their toxicity, as manifested by an intraperitoneal injection (*17*).

TABLE 1.—Hemagglutinins in rabbit anti-C3H spleen serum (tested against C3H, C57BL, and Swiss red blood cells)

Serum	Titer against red blood cells of:		
	C3H	C57BL	Swiss
Anti-C3H	128–256	128–256	512
Normal	—	—	—

We then tried to decrease the rate of emission of antibodies into the mouse blood-system by injecting the serum at the subcutaneous site. Nonabsorbed, inactivated antiserum, thus injected, killed all the mice, whereas absorbed antiserum *did not* have any detectable effect. The subcutaneous injection of absorbed antiserum was therefore adopted in all our experiments on enhancement by heteroimmune serum.

Since the absorptions carried out in the last experiments removed the complete agglutinins, and yet did not abolish the toxicity of the antiserum (produced after intraperitoneal injections), the absorbed antiserums were tested for incomplete agglutinins, by the dextran-human serum method (*13*) previously applied to test agglutinins elicited by

TABLE 2.—Incomplete (dextran) hemagglutinins in rabbit anti-mouse serum

Serum	Treatment of serum*	Tested against red blood cells of: C3H	C57BL	Swiss
Anti-C3H	Inactivated	>4096		
Anti-C3H	Inactivated and absorbed with C57BL red blood cells	>4096	4096	
Anti-C3H	Inactivated and absorbed with Swiss red blood cells	2048		>4096
Anti-C3H	Inactivated and absorbed with C3H red blood cells	>4096	>4096	>4096
Anti-C57BL	Inactivated and absorbed with C3H red blood cells	1024	1024	

*Heated 56° C. for 30 minutes to inactivate.

tissue homografts. It was found that the absorbed heteroimmune serum does contain incomplete agglutinins, showing a titer of >4096 (table 2).

Cytotoxins

The cytotoxic effect *in vitro* of heteroimmune serum was then tested, with both absorbed and unabsorbed serums. Cells of a number of ascites tumors, all of C3H origin (6C3HED, 6C3HED-ICR, MC1M, and MC1A), which vary in their *in vitro* susceptibility to cytotoxins of isoimmune serums (*15*), were used as test objects for cytotoxins in the heteroimmune serums. The results showed that the rabbit anti-C3H spleen serum contains antibodies which react with the tumor cells. This reactivity was manifested in (*a*) a cytotoxic effect, which is revealed through the application of trypan blue, (*b*) agglutination of the ascites tumor cells by the antiserum (without the addition of guinea-pig complement), and (*c*) swelling of cells, after complement is added *in vitro*.

The heteroantiserum showed a cytotoxic effect on cells of 6C3HED. The addition of complement to the antiserum increased their cytotoxicity, *i.e.*, the percentage of trypan-blue-positive cells (59% without complement, up to 89% with complement), and activated the cytotoxicity to MC1M cells (table 3). Cells of MC1A and 6C3HED-ICR were nonsusceptible to the cytotoxins, just as these are not susceptible to the effect of isoimmune serum (*15*). Similar results were obtained when inactivated serum (at 65° C. for 1 hour) was used with the addition of guinea-pig complement.

Absorption of the antiserum with homologous erythrocytes (of C57BL animals) did not markedly change the results (6C3HED: 95% in absorbed and 87% in unabsorbed; MC1M: 84% and 61%, respectively). Absorption with isologous C3H erythrocytes did, however, remove the cytotoxic activity against MC1M, though no marked changes were observed when antiserum was tested against 6C3HED (table 3).

When cytotoxicity was tested against normal C3H spleen cells, the results were similar to those obtained with 6C3HED cells (table 4). The unabsorbed antiserum showed 88 percent of trypan-blue-positive spleen cells, and neither the absorption with C57BL nor with C3H erythrocytes

TABLE 3.—Cytotoxic activity *in vitro* of heteroimmune serum on ascites tumor cells

Serum tested	Treatment of serum			Tumor cells tested*			
	Inactivation 65° for 60′	Absorbed with	Addition of complement	MC1M	MC1A	6C3HED	6C3HED-ICR
Anti-C3H	−	—	+	75. 4	3. 1	89. 3	9. 4
Anti-C3H	−	—	−	46. 2	13. 2	59. 6	
Anti-C3H	+	—	+	84. 8	4. 5	87. 1	14. 3
Anti-C3H	+	—	−	20. 6	5. 7	22. 7	4. 3
Anti-C3H	+	C3H RBC	+	27. 5		83. 0	
Anti-C3H	+	C3H RBC	−	4. 5		8. 0	
Anti-C3H	+	C57BL RBC	+	61. 5		95. 0	
Anti-C3H	+	C57BL RBC	−	2. 5		8. 0	
Anti-C3H	+	C3H liver cells	+	31. 5	12. 2	14. 3	14. 2
Anti-C3H	+	C3H liver cells	−	30. 1	8. 8	21. 8	16. 6
Anti-C3H	+	C57BL liver cells	+	27. 6	13. 7	75. 4	11. 5
Anti-C3H	+	C57BL liver cells	−	40. 6	12. 5	36. 2	29. 3
Normal	+	—	−	20. 4	3. 1	1. 4	3. 5
Normal	+	—	+	26. 0	3. 0	16. 3	6. 3

*Percent of stained cells.

TABLE 4.—Cytotoxic activity *in vitro* of heteroimmune serum on normal spleen cells

Serum tested	Treatment of serum			Spleen cells tested	
	Inactivation 60° for 60′	Absorbed with	Addition of complement	C3H	C57BL
Anti-C3H	−	—	+	87. 0	97. 0
Anti-C3H	−	—	−	65. 0	71. 0
Normal	−	—	+	48. 5	24. 0
Normal	−	—	−	10. 0	6. 0
Anti-C3H	+	—	+	88. 0	98. 0
Anti-C3H	+	—	−	19. 0	20. 5
Normal	+	—	+	38. 0	22. 5
Normal	+	—	−	6. 0	6. 0
Anti-C3H	+	C3H RBC	+	89. 0	91. 5
Anti-C3H	+	C3H RBC	−	17. 0	5. 0
Anti-C3H	+	C57BL RBC	+	94. 0	90. 0
Anti-C3H	+	C57BL RBC	−	7. 0	5. 0

changed the cytotoxicity. This is reconcilable with the cytotoxicity *in vivo* produced by the intraperitoneal injection of absorbed antiserum. Since the 6C3HED cells behaved, in this respect, like the normal C3H cells, we used the lymphoma cells for a further test for strain-specific cytotoxins.

We then tried to remove the cytotoxic activity against 6C3HED cells by absorbing antiserum with nucleated cells, as distinct from red blood cells, of mice. The antiserums were therefore absorbed with liver cells of isologous C3H and homologous C57BL animals. These two absorptions removed the cytotoxic activity against MC1M cells, but against 6C3HED cells it was removed only in the sample that had been absorbed with C3H liver cells (table 3). It thus appears that the heteroimmune serum against C3H cells does contain antibodies specific to the C3H isoantigens, in addition to those which react with nonrelated strains.

Enhancement of Homografts of 6C3HED Lymphosarcoma by Anti-C3H Serum

Tests *in vitro* showed that the cells of 6C3HED lymphosarcoma are susceptible to the cytotoxic action of anti-C3H serum. Although cytotoxic *in vitro*, such antibodies in isoimmune serum are not responsible for the cytotoxic effect *in vivo*, *i.e.*, for the destruction of the tumor homografts (*15*). Hence it was of interest to test the effect *in vivo* of heteroimmune serum on homografts of this tumor.

In one group, each of the 6 C57BL mice was injected with 0.5 cc. of rabbit anti-C3H serum, and 1 hour later the animals were injected intraperitoneally with 6C3HED cells. The tumors in this group grew for 19 days before regression (one resulting in a lethal take), whereas in untreated control C57BL mice, they regressed within 12 days. On the 16th day, a sample of ascites cells from the C57BL hosts was tested for viability by reinoculating the cells into C3H animals. The latter were killed by the tumor on the 9th day, as with normal C3H to C3H grafts.

In a second group of 5 C57BL mice the treatment with antiserum plus

tumor inoculation resulted in lethal takes, deaths occurring on the 6th, 11th, and 13th day.

A third group of 5 C57BL mice was similarly treated, and the 6C3HED homografts showed a lethal effect between the 8th and the 12th days. In all control C57BL animals the tumor was rejected within 12 days.

These results thus indicate that anti-C3H serum enhances the growth of 6C3HED homografts, and induces, in most cases, lethal takes of an otherwise nontransplantable tumor.

To test whether the "enhanced" growth of the 6C3HED homografts is due to the inactivation of the immune response of the host, or whether the induced enhancement takes place *in spite* of the host's response, the isohemagglutinins produced in the homograft reaction were tested. Serums of treated animals were tested against C3H red blood cells, in comparison to control serums of C57BL inoculated with 6C3HED cells without the injection of antiserum. Bleedings were made on the 8th and 11th day after tumor inoculation. In both groups a similar titer of isohemagglutinins was found (1:32–1:64). Although these circulating antibodies are not directly involved in transplantation immunity, their production indicates that the enhanced tumor growth does elicit a homograft reaction in the foreign host.

Enhancement of Homografts of Sarcoma SBL1 by Anti-C57BL Serum

In further experiments on the induction of enhancement, anti-C57BL serum was tested for its ability to induce growth of Sarcoma SBL1 in C3H mice.

The antiserum, absorbed with C3H red blood cells, was injected (0.5 ml. per mouse) into two groups of 5 and 6 mice each. One hour later the mice were grafted subcutaneously with Sarcoma SBL1. In the antiserum-treated animals the tumors grew for a prolonged time and attained a larger size than in the control mice. The "enhanced" SBL1 grew progressively for 21 to 32 days and the tumors developed necrotic lesions and were eventually "shed off" by the host (table 5). In one animal the tumor grew for 60 days and was then tested for viability by retransplantation into C57BL mice; all the grafts took. In the controls the grafts showed only an initial growth, attaining a size of a few millimeters, and regressed completely on the 12th to 16th day. Anti-C57BL serum thus enhances the growth of C57BL tumors in a foreign strain.

To test whether the site of a tumor graft in relation to the site of an antiserum injection might influence the enhancing effect, a further experiment was carried out, in which the antiserum was injected subcutaneously and the tumor grafted intramuscularly. The results showed (table 5) that in this case also an enhancing effect was produced by the antiserum.

To test whether the enhancement of Sarcoma SBL1 in a foreign strain is due to normal components of rabbit serum serving as "growth substances," C3H mice were injected with 0.5 ml. of normal rabbit serum; one group received grafts of Sarcoma SBL1 subcutaneously and the other intramuscularly. No differences in tumor growth were found between

TABLE 5.—Enhancement of SBL1 in C3H mice treated with antiserum

Host's strain	Number of animals	Treatment of hosts	Site of tumor graft	Growth until regression		Mortality	Days of mortality
				Days	Number of animals		
C3H	10	Anti-C57BL	Subcutaneous	21	2/10	1/10	28
				22	1/10		
				24	2/10		
				25	3/10		
				60	1/10*		
C3H	14	—	Subcutaneous	12	10/14	0/14	—
				14	4/14		
C57BL	10	—	Subcutaneous			10/10	17—24
C3H	7	Anti-C57BL	Intramuscular	18	1/7	—	—
				23	1/7		
				27	3/7		
				31	2/7		
C3H	12	—	Intramuscular	13	7/12	—	—
				14	2/12		
				16	3/12		

* One animal was killed after 60 days of tumor growth, for retransplantation.

these and untreated animals. This confirms previous indications that tumor enhancement by antiserum should be attributed to the action of antibodies.

The Mechanism of Enhancing Action of Antibodies

The enhancing action of antibodies was, as indicated, attributed to the following two main possibilities:

1) The antibodies inhibit the production of transplantation immunity by the host, because they cross-react with the transplantation isoantigens of the tumor cells (*5*, *6*). The latter are neutralized by the antibodies, which thus prevent the production of an immune response of the host.

2) The antibodies act directly on the tumor cells and induce some "physiological" changes in the tumor, which ensure its progressive growth (*1*, *8*).

The following experiments were designed to test these two possibilities.

It was previously shown that the growth of an enhanced homograft of 6C3HED is *not* accompanied by a suppressed production of anti-C3H agglutinins. Since these hemagglutinins are not directly involved in transplantation immunity, further experiments tested whether, during the progressive growth of a tumor homograft enhanced by antiserum, the tumor elicits a heightened host resistance, *i.e.*, transplantation immunity.

1. *Active immunization by enhanced tumors.*—These experiments were based on the following assumption: If during the progressive growth of an enhanced tumor, the foreign host is not actively immunized by the SBL1 antigens, then the reinoculation of the same animals with a second SBL1 graft, following the extirpation of the first enhanced SBL1, should

not reveal any secondary response, whereas the extirpation of a normal SBL1 homograft, followed by a second SBL1 graft, would reveal a clear secondary response.

Two groups of 8 C3H mice, each, were grafted subcutaneously with SBL1 (table 6, groups 1 and 2). The animals of group 1 were treated with anti-C57BL serum. Seven days later the tumors of both groups were extirpated under ether anesthesia, and 3 days after tumorectomy, all the animals were regrafted with SBL1. The results are given in table 6. In the control group, the second SBL1 graft, after the extirpation of the first SBL1, did not show any signs of growth. In the experimental group, 7 of 8 also did not show any tumor growth. In one animal, however, the tumor developed for 28 days and attained a diameter of 13 mm., but this was most probably a remnant of the first enhanced graft, which was not completely removed. It is therefore concluded that the enhanced SBL1, while growing progressively in antiserum-treated mice, does elicit the production of transplantation immunity. The growth of an "enhanced" tumor homograft takes place *in spite* of the immune response it develops.

TABLE 6.—Active immunization by enhanced and normal homografts of SBL1

Experimental group No.	First homograft	Second homograft	Number of animals developing tumors	Growth until regression (*days*)	Mortality
1	SBL1+ antiserum	SBL1	1/8	28	—
2	SBL1	SBL1	0/8	—	—
3	SBL1	SBL1+ antiserum	0/8	—	—
4	SBL1+ antiserum	SBL1+ antiserum	8/8	19 (7/8)	1/8 (70 days)
Control	—	SBL1	8/8	13—16	—

We then tested whether the active immunization produced during the growth of an enhanced SBL1 (=SBL1e) is reflected also in the production of anti-C57BL isohemagglutinins, like the production of anti-C3H agglutinins in C57BL mice carrying an enhanced 6C3HED tumor. Serums of two groups of C3H animals grafted with SBL1, but only one receiving treatment with antiserum, were tested against C57BL red blood cells. The animals were bled on the 8th and 11th day following tumor grafting. Neither group showed any production of agglutinins. Thus transplantation immunity produced routinely or during enhancement by antiserum need not involve an "agglutinin response."

2. *The intensity of the immune response elicited by enhanced SBL1.*—The difference between the intensity of the immune response produced by an ordinary and an enhanced SBL1 (=SBL1e) homograft was tested in the following experiment: In one group, 8 C3H mice were grafted with SBL1

(table 6, group 3). A second group (group 4) was grafted with SBL1 and received an injection of anti-C57BL serum. The tumors of both groups were extirpated on the 7th day, and 3 days later the mice were regrafted with SBL1 and received an injection of antiserum (=SBL1e). The results (table 6) showed that the animals first immunized with SBL1 did not show any signs of growth of the second SBL1e, whereas in those that were preimmunized with SBL1e, the second SBL1e graft did develop in all of them. In 7 of 8, regression of the tumors started on the 19th day, and one animal died of a progressive tumor after 70 days.

Thus a second enhanced graft (SBL1e) can overcome the host's resistance produced by the first enhanced tumor, but not that of a first ordinary SBL1 graft. Hence, though both SBL1 and SBL1e elicit transplantation immunity, the intensity of host resistance elicited by one enhanced SBL1e graft is lower than that elicited by SBL1.

This, however, does not mean that the actual "antigen emission" of SBL1e is lower than that of SBL1. It seems probable that exposure of the C3H hosts, in the experimental group, to both tumor antigens and the rabbit-serum antigens, might account for the relatively lower response to the cellular antigens of the tumor homograft. Simultaneous exposure of animals to a heterogeneous system of antigens might cause a relatively lower immune response to each of them [*see* Discussion in (*9*)].

3. *The resistance to transplantation immunity of a SBL1e tumor.*—The concept that the progressive growth of an enhanced SBL1 (=SBL1e) tumor should be attributed to its ability to resist the transplantation immunity it produces, and not to its decreased functional antigenicity, was manifested further in experiments on passive immunization.

Forty-eight C3H mice were challenged with 2 lateral subcutaneous grafts of SBL1 and 1 subcutaneous injection of antiserum. Seven days later, the inguinal, brachial, and axillar lymph nodes (6 nodes from each animal) were excised, minced, and then injected intraperitoneally, 18 nodes to each host, into 2 groups of 8 C3H animals each. Three days after the implantation of the lymph nodes, one group received a graft of SBL1 and the other SBL1 plus an antiserum injection (=SBL1e). None of the passively immunized animals receiving a graft of SBL1 showed any signs of tumor growth. In the passively immunized animals challenged with SBL1e, all developed tumors. Three of 8 mice showed only slight growth up to the 10th day, but in 4 of 8, the tumors did not regress until the 17th day, and in 1 animal regression took place only after the 22d day. These findings thus show that the immune response elicited by the subcutaneous SBL1e was high enough to confer adoptive immunity in normal C3H mice and to prevent any growth of SBL1 in these animals. Homografts of SBL1e, however, could at least partially overcome this adoptive immunity.

The experiments reported indicate that the enhanced homograft (*a*) does elicit a homograft reaction and (*b*) can resist the homograft reaction it produces. It can therefore be deduced that the injected antibodies did act on the tumor cells and made the latter capable of resisting trans-

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The experiments reported indicate that the enhanced homograft (*a*) does elicit a homograft reaction and (*b*) can resist the homograft reaction it produces. It can therefore be deduced that the injected antibodies did act on the tumor cells and made the latter capable of resisting trans-

plantation immunity. This implies a direct action of the antibodies on the tumor cells. In previous studies in this laboratory (*9*), resistance of certain homotransplantable tumors to the homograft reaction was attributed to increased antigen synthesis. The antigen excess formed neutralizes extracellularly the "cytotoxic antibodies" of the homograft reaction and thus prevents the antibodies from destroying the tumor cells. If enhancing antibodies act directly on the tumor cells, and stimulate in the latter a more intense synthetic processes, this might explain the resistance of enhanced tumors on the basis of antigen excess.

If, however, enhancement is attributed to a direct stimulating action of the antibodies on the tumor cells, one would expect a similar stimulating effect of "enhanced growth" of the tumor in animals of the tumor's isologous strain, if the latter are injected with antibodies. To test this, C57BL mice each received an injection of anti-C57BL serum and were challenged with a subcutaneous isograft of SBL1. Similar grafts were made in 8 untreated C57BL animals. It was observed that in the antiserum-treated mice, the growth of the tumor grafts was enhanced (table 7). At 13 days after tumor transplantation, the mean diameter of the tumors in the experimental group was 17.5 mm., whereas in the untreated animals it was 13.0 mm., and at 23 days it was 32.0 mm. versus 25.0 mm. in the controls. Thus a clear indication of enhancement of tumor *isografts* by antiserum was obtained in these experiments. Since no isoimmune response is conceived in these animals, the results cannot be explained by a suppression of the host's resistance. They therefore support the concept of a direct action of the antibodies on the tumor cells, stimulating in the latter an augmented growth. That the relative enhancement obtained in this experiment is smaller than that of enhanced homografts is probably because most of the antibody injected into the C57BL animals was absorbed by the somatic tissues of the isologous mice, unlike that of the anti-C57BL serum when injected into C3H animals.

Enhancement of SBL1 by Anti-C3H

Since tests *in vitro* have shown that rabbit anti-C57BL serum contains incomplete hemagglutinins against C3H red blood cells, an experiment was undertaken to test whether anti-C3H serum has an enhancing effect on SBL1 homografts. Two groups of C3H animals received an injection of anti-C3H serum, and one group received a SBL1 graft subcutaneously and the other intramuscularly. In both groups clear indications for an enhancing effect were obtained (table 8).

Enhancement of MC1M by Lymph Nodes of Immunized Animals

Passive transfer of transplantation immunity, *i.e.*, adoptive immunity, can be accomplished only by activated lymph-node cells (*18*). Serum antibodies from immunized animals do not confer immunity on normal animals except in a few cases of leukemia (*19, 20*). Enhancement was passively transferred to normal animals by circulating antibodies of either

TABLE 7.—Effect of antiserum on growth of SBL1 isografts in C57BL mice

Days following tumor transplantation	SBL1 + antiserum		SBL1	
	Number of tumor-bearing animals	Mean diameter of tumor (*mm.*)	Number of tumor-bearing animals	Mean diameter of tumor (*mm.*)
7	6/8	7.0 ± 0.32	6/7	6.0 ± 0.13
9	6/8	11.0 ± 1.1	6/7	8.8 ± 0.23
10	6/8	11.5 ± 0.25	6/7	10.0 ± 0.23
12	6/8	16.2 ± 1.58		12.5 ± 0.9
13	6/8	17.5 ± 2.0		13.0 ± 0.65
14	5/7*	18.5 ± 2.1		15.0 ± 1.25
16	5/7	21.5 ± 3.0		18.0 ± 1.25
23	5/7	32.0 ± 3.0		25.0 ± 0.23

*One died with necrotic tumor.

TABLE 8.—Enhancement of SBL1 in C3H mice treated with rabbit anti-C3H serum

Treatment	Site of tumor graft	Number of animals	Growth until regression		Maximal diameter of tumor (*mm.*)	Mortality
			Number of animals	Days		
Injected with antiserum	Subcutaneous	6	2	17	20–30	1/6
			1	19		
			1	24		
			1	29		
	Intramuscular	5	1	11		0/5
			2	16		
			1	20		
			1	22		
	Subcutaneous	9	3	12	5	0/9
			5	14		
			1	16		
Untreated	Intramuscular	5	2	0*		0/5
			1	14		
			1	16		
			1	17		

*Regressed without developing palpable tumor.

isoimmune or heteroimmune serums. Previous studies by Kaliss have shown that active enhancement can be induced by prolonged immunization with tumor homografts (*8*). When such homografts elicit transplantation immunity, the regional draining lymph nodes are mainly involved in the immune response. It seemed of interest, therefore, to test whether enhancement induced by homografts can also be passively transferred by the lymph-node cells, *i.e.*, whether the same lymph nodes that normally produce transplantation immunity would, after a prolonged immunization by tumor homografts, produce the enhancing antibodies. To test this, we used the MC1M ascites tumor in the following experiments.

Nine groups of 21 Swiss mice, each, received 2 simultaneous lateral subcutaneous injections of MC1M ascitic fluid (15×10^6 cells). At various intervals after the tumor inoculations, the animals were killed

and the inguinal, axillary, and brachial lymph nodes were excised, minced, and then injected into other Swiss mice. The nodes from each group were injected intraperitoneally into 7 animals, *i.e.*, from 3 donors to 1 host. One or 3 days after the injection of lymph-node cells, the animals were injected intraperitoneally with MC1M. The results were as follows: Activated lymph-node cells transferred 2, 3, and 4 days after immunization of the donors did not confer any immunity. The hosts developed tumors as in untreated controls. Lymph-node cells transferred 5 days after immunization of the donors conferred transplantation immunity; the intraperitoneal MC1M homografts were rejected. But, lymph-node cells transferred 9, 12, and 15 days after immunization conferred enhancement: The tumors grew more rapidly than in untreated animals, and death from tumor progression occurred earlier than in untreated controls (table 9). Mice inoculated with lymph nodes 9 days after immunization showed an increase in weight per animal of 3.3 gm. on the 7th to 9th day of tumor growth, whereas untreated controls gained only 0.8 gm. per animal, due to the growth of MC1M (table 10).

It is therefore concluded that (*a*) the same lymph nodes shown to confer transplantation immunity can confer, under certain conditions, immunological enhancement, and (*b*) one of the conditions required for the

TABLE 9.—Passive transfer of enhancement of MC1M and of transplantation immunity against MC1M by activated lymph-node cells

Activation of donor's lymph nodes (*in days*)	Mortality due to tumor growth					
	Experimental			Control (*normal*)		
	Days	Number of animals	Total mortality	Days	Number of animals	Total mortality
3	10	2/7	7/7	5	1/7	7/7
	14	1/7		18	4/7	
	16	3/7		21	1/7	
	18	1/7		25	1/7	
5	19	1/7	1/7	9	1/7	7/7
				17	5/7	
				21	1/7	
9	7	1/7	5/7	16	3/7	7/7
	10	1/7		17	1/7	
	12	1/7		18	1/7	
	15	1/7		19	2/7	
	16	1/7				

TABLE 10.—Increase in weight of MC1M-bearing animals inoculated with lymph nodes that were immunologically activated for 9 days

Weight of animals (*in gm.*)	Experimental	Control *
At 0-time	24 ± 0.7	25 ± 0.25
9 days after tumor inoculation	27.5 ± 1.1	25.8 ± 0.94
Mean of weight increase	3.5	0.8

*Inoculated with MC1M without lymph-node cells.

enhancing response is a relatively prolonged immunization. In view of these changes in the type of immunological reaction manifested by the same lymph nodes, a further experiment was made to test the active (as distinct from passive) immune response at sites remote from the regional lymph nodes. Swiss mice were given two lateral, subcutaneous inoculations of MC1M, and 9 days after tumor transplantation, the "regional" inguinal, brachial, and axillar lymph nodes were excised under ether anesthesia. One day later, the same animals were given an intraperitoneal inoculation of MC1M. A control group of nonimmunized Swiss mice was similarly subjected to a removal of the normal lymph nodes and, 1 day later, inoculated with an intraperitoneal graft of MC1M. Whereas in the controls all the mice showed a lethal take of the intraperitoneal homograft, all the experimental animals rejected the MC1M cells. The host resistance shown in the experimental group can be attributed to the fact that the "nonregional" peritoneal lymphatic nodules, at a remote site in relation to the first immunizing tumor graft, are, in fact, exposed to the antigens of the tumor for a shorter functional time; and while the regional draining lymph nodes are already producing enhancing antibodies, those of the peritoneum are still producing transplantation immunity. Thus the response of an animal after active immunization with tumor homografts might differ from the response of "adoptively" immunized animals and is determined by the relative intensity of the two types of immune responses.

DISCUSSION

Types of Antibodies in the Enhancing Antiserum

The present study provides further evidence that the active factors responsible for enhancing tumor homografts are humoral antibodies. The main role in the induction of tumor enhancement was attributed to the isohemagglutinins of the *H-2* system (*5*). This conclusion was derived from the apparent correlation between the titer of agglutinins and the effect of enhancement. In the serums we used for enhancing tumor homografts, a number of antibody types were demonstrated: 1) complete agglutinins, 2) incomplete agglutinins, 3) cytotoxic antibodies, and 4) precipitin antibodies, reacting with saline extracts of the homologous normal-tissue homogenates (unpublished data). It appears from our present experiments that the complete agglutinins are not involved in the enhancement phenomenon, which could be derived from the enhancing effect of isoantiserum, but it is impossible, at the present stage of our work, to determine which of the other types of antibodies, including those yet undetermined, are the active factors in enhancing tumor homografts. In arguing as to the possible mechanism of action of antibodies, one might attribute to the cytotoxins (activated *in vitro* by guinea-pig complement) a determining role in tumor enhancement: If these cytotoxic antibodies affect only part of the tumor cell population, the latter might release sub-

stances which act as growth promoters on the viable unaffected cells. However, since inactivated and absorbed anti-C3H serum is cytotoxic *in vitro* to 6C3HED cells only if supplemented by guinea-pig complement, and since these antibodies do not display cytotoxicity *in vivo* (*15*), it seems very improbable that the enhancing effect is based simply on a direct injury to the tumor cells.

Snell has maintained that antigens determined by the *H-2* complex are responsible for eliciting the enhancing antibodies (*21*). We found that both anti-C3H and anti-C57BL heteroantiserums contain incomplete agglutinins (activated by dextran and human serum) against C57BL erythrocytes. Such antibodies, demonstrated in serums absorbed with red blood cells, may represent the isohemagglutinins produced by isoimmunization and directed against the *H-2* agglutinogens. Therefore one would expect that both anti-C57BL and anti-C3H serums would enhance SBL1 homografts, as observed in our experiments. But these two stains contain one common antigen of the *H-2* system—the E antigen (CdE_tK in C3H and cDEFk in C57BL). Thus if the *H-2* antigens determine the enhancing response, it appears that anti-E might be one of the active components involved in the enhancing effect. It should, however, be stressed that even if the specificity of the enhancement phenomenon derives from the *H-2* antigens, there is as yet no evidence that the agglutinins, as distinct from other types of antibodies, are the active factors.

Mechanism of Action

The experiments reported have shown clearly that the enhancing antibodies do not prevent the production of a homograft reaction against the enhanced tumors. Homografts of normal and enhanced 6C3HED cells elicited the same level of isohemagglutinins. Furthermore, enhanced SBL1 was shown to produce transplantation immunity (host-resistant), which was demonstrated both by active immunity and passive transfer of transplantation immunity. The fact that anti-C57BL serum enhanced SBL1 isografts in C57BL hosts shows also that the mechanism of enhancement is not based on the prevention of the hosts' resistance, since the SBL1 has originated in the same stock of C57BL mice. It thus appears that the enhancing antibodies act directly on the tumor cells (*1*). When the tumor is indigenous to the host, the change produced by the enhancing antibodies is manifested in accelerated growth of the isograft. When the tumor is genetically foreign to the host, this change is manifested in the capacity of the tumor cells to resist the transplantation immunity which they themselves evoke, while growing progressively in a noncompatible environment. These two manifestations of enhancement of isografts and homografts *should* have a common mechanism. It has been previously claimed (*9*) that the resistance of homotransplantable tumors to the homograft reaction is based on an intensified production of antigens by the resisting tumors. The intense production of antigens might enable an efficient neutralization of the "cytotoxic" antibodies produced by the resisting host. The induction of an increased synthesis of antigens would

obviously explain the enhancement of tumor homografts, but might also imply the accelerated growth of tumor isografts if the antigens are somehow connected with nuclear constituents, as they are claimed to be (*21*), even without the necessary participation of DNA in the transplantation antigens (*22*). How, then, can antibodies elicit in tumor cells an augmented production of antigens? Obviously, at this stage any elaboration on this question would be sheer speculation. Yet we should like to point out two alternative possibilities that might furnish working hypotheses for a further analysis:

a) The "enhancing" antibodies are elicited by and react with inhibitors connected with the synthesis of the transplantation antigens. According to this, the enhancing antibodies stimulate indirectly the production of *H-2* antigens.

b) The antibodies, elicited by *H-2* antigens, directly stimulate the production of their homologous antigens. Such a stimulation could be visualized by assuming that certain *types* of antibodies produced by cellular antigens can elicit in the antigen-carrying cells (*i.e.*, in the cells of the tumor homograft) an augmented synthesis of the homologous antigen, through *a mechanism similar to that by which the antigen elicits in the lymph-node cells the synthesis of the homologous antibody*. The latter reaction, *i.e.*, the formation of antibodies, which seems to involve the participation of RNA (*23*), and most probably also of DNA, is triggered by the antigen and can be designated by the following simplified scheme:

$$\text{Antigen} \rightarrow \text{RNA} \rightarrow \text{Antibody}$$

The induction of enhancement by antibodies would then be a reciprocal reaction, taking place in the tumor cell and triggered by the antibody. It could, accordingly, be designated as follows:

$$\text{Antigen} \leftarrow \text{RNA} \leftarrow \text{Antibody}$$

This last assumption implies that after a prolonged immunization the *H-2* antigens form new types of antibody, which, when attached to the tumor cells, can stimulate a reciprocal reaction of synthesis of the homologous antigen. There is evidence that by prolonged immunization, different types of antibodies might be elicited by the same antigen. However, since the second hypothesis introduces a still unknown type of immune reaction, its verification must await a direct demonstration of the reciprocal reaction.

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Chromosomes of Tumor Cells. I. Murine Leukemias Induced by One or Two Injections of 7,12-Dimethylbenz[*a*]anthracene [1]

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SUMMARY

One or two injections of 7,12-dimethylbenz[*a*]anthracene (DMBA) into baby mice of the low-leukemic Swiss stock resulted in a high incidence of lymphatic leukemias. The first mouse, which died at 65 days of age, had a thymic lymphosarcoma, greatly enlarged spleen and lymph nodes, and infiltration of leukemic cells in the kidneys and lungs. Four months after DMBA injection 50 percent of all mice developed leukemias, which were transplantable to adults of the same mouse strain. The chromosome number was studied in thymic lymphosarcomas of 16 mice and in various infiltrated organs of 3 mice. Fifteen of the 16 thymic lymphosarcomas consisted of a population of cells containing predominantly 41 chromosomes, as compared with 40 chromosomes in normal tissues. There were also aneuploid cells in the infiltrated spleen and lymph nodes. Cells having predominantly 42 chromosomes were observed in the enlarged thymus, spleen, and lymph nodes of one mouse. The preference of one aneuploid chromosome number in the thymic lymphosarcomas is discussed in regard to formation and selection of the neoplastic cell.—J. Nat. Cancer Inst. 25: 649–661, 1960.

THE REGULAR appearance of abnormal chromosome complements in neoplastic cells supports the hypothesis that alterations in the genotype are involved in the transformation of normal cells into malignant ones. Assuming that a particular chromosome or gene combination is responsible for the malignant properties of a tumor cell, it should be expected that tumors of a particular type would have similar chromosome abnormalities. Translocation, inversions, and the presence of chromosomes not essential for the malignant properties but also not lost during the selection processes might introduce a certain variability of the aneuploid chromosome complement, thus obscuring a basic pattern. At present comparative cytological data on one tumor type are so scarce that these postulations can be neither confirmed nor discarded. The shift of a number of different mouse ascites tumors to a hypotetraploid chromosome number (*1*), the accumulation of deoxyribonucleic acid (DNA) values of human intestinal adenocarcinomas in a hyperdiploid and hypotetraploid

[1] Received for publication March 2, 1960.

range (*2, 3*), and a relatively small scatter in radiation-induced leukemias (*4*) are the only observations indicating a certain regularity.

The present study was undertaken to gain information as to whether all tumors would show chromosomal changes, and if so, whether a particular tumor is characterized by a particular chromosome pattern. Lymphatic leukemias induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in mice were chosen for the present study (*5*). The relatively large size of the leukemic cells and the softness of the lymphatic tissue are suitable for chromosome studies. The opportunity to compare the result with those obtained on radiation-induced (*4*) and spontaneously occurring leukemias (*6, 7*) is of further advantage because it permits comparison of the chromosome abnormalities caused by different carcinogenic agents.

MATERIALS AND METHODS

Mice of the Swiss stock obtained from Connaught Laboratories (Toronto) and further bred at random in this laboratory were used throughout the experiments. A colloidal suspension of 7,12-dimethylbenz[*a*]anthracene (DMBA), which was prepared according to the technique of Pietra *et al.* (*5*), was injected subcutaneously in the interscapular region of baby mice less than 24 hours of age. Each mouse received 0.03 ml. of this suspension, which contained 60 μg. DMBA. Small droplets of the DMBA suspension, however, sometimes appeared at the puncture site and were lost. Thus the baby mice did not obtain exactly the same amount of DMBA. Mice given a second injection received the same amount of DMBA subcutaneously 7 days after the first injection.

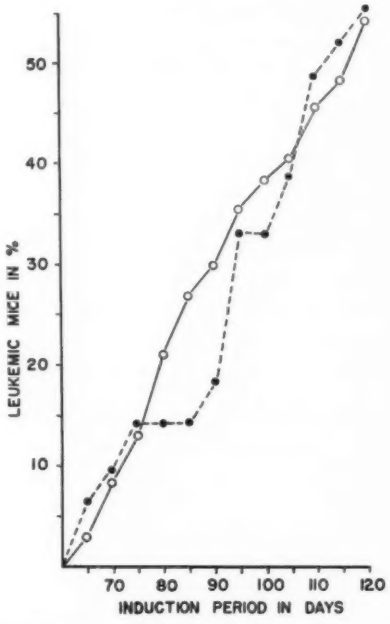
Transplantations were performed by grafting, subcutaneously, 1 to 2 mm. pieces of thymic lymphosarcomas or infiltrated mesenteric lymph nodes by the trocar technique. Recipients were adult mice of the Swiss stock 2 to 4 months old.

Leukemic mice were recognized by a characteristic deep breathing, by an enlarged spleen, which could be palpated, by slow movements, and frequently by swollen cervical or axillary lymph nodes. These mice, when moribund, received 0.8 ml. intraperitoneal injections of a 0.2 percent colchicine solution and were killed 1 to 1½ hours later. The leukemic tissues were removed, chopped into small pieces not exceeding 1 mm. in diameter, pretreated with a 1.36 percent sodium citrate solution for 20 to 30 minutes at 37° C. (*8*), and placed into a 1 percent orcein-HCl solution. Squash preparations were generally made of tissues stained for 2 to 4 hours, and then sealed with "Deckglaskit" of Kröning (*9*), which kept them in satisfactory condition for several weeks. A phase-contrast microscope was used for cytological examinations. The length of chromosomes was determined by using a microscale in the ocular (20×) or on greatly enlarged projections of photomicrographs of metaphase plates.

RESULTS

Incidence of Leukemia

The incidence and latent period of leukemias induced by 1 or 2 subcutaneous injections of DMBA are shown in text-figure 1. Animals used for calculating the percentage of leukemias during a 4-month period either died or were killed when moribund. The presence of leukemia was diagnosed macroscopically and microscopically. Mice that died between 3 and 5 weeks of age were excluded from the calculations. The growth of these mice was retarded and the liver usually exhibited a fatty degeneration, whereas the thymus, spleen, and lymph nodes appeared macroscopically normal. No leukemias developed in normal mice of the Swiss stock used in this laboratory during the first 4 months of age.



TEXT-FIGURE 1.—Induction of leukemias due to injection of 7,12-dimethylbenz-[a]anthracene. ○ = 1 injection; ● = 2 injections.

Pathology

Macroscopically the organs and tissues of the leukemic mice were surprisingly uniform. The thymus was greatly enlarged in all the moribund specimens (fig. 1), and their weight varied from 0.7 to 1.2 gm. as compared with 0.05 to 0.1 gm. in normal mice of corresponding age. The spleen was enlarged in 49 of 55 leukemic mice, although the size and weight varied greatly: 6 spleens had a weight of 2 to 3 gm. (fig. 2), 11

from 1 to 2 gm., 21 from 0.5 to 1 gm., and 11 from 0.4 to 0.5 gm. There was marked enlargement of lymph nodes in 52 of 55 leukemic mice, but infiltration of leukemic cells in the lymph nodes varied. The thoracic, mesenteric, and cervical lymph nodes frequently were enlarged, the axillary lymph nodes were involved to a lesser degree, and the inguinal, lumbar, and caudal lymph nodes were occasionally, and then only slightly, swollen.

Microscopic examination showed the presence of leukemic cells having a relatively large, round nucleus and a scanty cytoplasm in the thymus, spleen, and lymph nodes, which obliterated the normal histological structure of these organs (fig. 3). Cells of the same type infiltrated, to varying degrees, the kidney (fig. 4) and lung (fig. 5). The leukemic cells in the liver accumulate around the blood vessels (fig. 6).

Twenty-eight out of 45 subcutaneous grafts of small pieces of tissue taken from thymic lymphosarcomas or infiltrated lymph nodes in adults of the same mouse strain developed into subcutaneous lymphosarcoma at the site of grafting, caused a general leukemia with infiltration in various organs, or resulted in both—generally in a period of 2 to 3 months.

Chromosome Number

Lymphosarcomas of the thymus, which developed 9 to 16 weeks after the first DMBA injection, were used for cytological examinations. The chromosome numbers observed in cells of thymic lymphosarcomas of 16 leukemic mice are summarized in table 1. Fifteen thymic lymphosarcomas consisted of cells having predominantly 41 chromosomes (figs. 7–10 and 12–18), and the enlarged thymus of one leukemic mouse, #8, contained predominantly cells with 42 chromosomes (fig. 11). Polyploid cells were

TABLE 1.—Number of chromosomes in lymphosarcomas of the thymus induced by DMBA

Mouse No.	Sex	Age of mouse when killed (*in days*)	Number of cells with indicated chromosome numbers						Total No. of cells
			39	40	41	42	43	80–84	
Control	F	65	4	47					51
1	M	65	—	4	27	—			31
2	M	72	2	4	20	4			30
3	F	72	—	3	24	8			35
4	M	74	1	6	19	2	2	—	30
5	M	87	2	7	20	2			31
6	F	90	2	6	20	2			30
7	M	91	1	5	23	1			30
8	F	93	—	10	9	28	—	4	51
9	M	94	—	5	20	5			30
10	M	100	—	2	21	7			30
11	M	106	1		28	1			30
12	F	106	1	12	26	1	1	—	41
13	M	106	1	5	22	3	—	11	42
14	F	106	2	4	26	11	—	6	38
15	F	107	—	3	20	7	—	—	30
16	F	108	1	4	34	1	2	—	42

TABLE 2.—Number of chromosomes in various infiltrated organs of 3 leukemic mice

Mouse No.	Sex	Organ	Number of cells with indicated chromosome number					Total No. of cells
			39	40	41	42	80–84	
1	M	Thymus	—	4	27	—	—	31
		Spleen	—	21	11	3	—	35
		Mesenteric lymph nodes	—	6	26	—	—	32
6	F	Thymus	2	6	20	2	—	30
		Mesenteric lymph nodes	—	5	21	2	—	28
		Cervical lymph nodes	1	7	21	3	—	32
		Axillary lymph nodes	—	6	11	—	—	17
8	F	Thymus	—	10	9	28	4	51
		Spleen	—	8	5	15	6	34
		Cervical lymph nodes	—	4	1	10	3	18
		Axillary lymph nodes	—	6	4	18	—	28

observed in mouse #8, #13, and #14, for which the chromosome number is given as 80 to 84. The chromosome count obtained on thymic cells of a normal 9-week-old Swiss mouse is included as control in table 1.

Chromosome counts obtained from various infiltrated lymph nodes and enlarged spleens of 3 leukemic mice are shown in table 2. Two of these mice (#1 and #6) had predominantly 41 chromosomes in cells of the thymic lymphosarcomas and showed the same chromosome number in the other leukemic tissues. Cells with 42 chromosomes were most frequently observed in the enlarged thymus of mouse #8, and the preference for this chromosome number was observed in infiltrated spleen and cervical and axillary lymph nodes of the same mouse.

A single small chromosome resembling the Y chromosome in shape and size (*10*) was observed in a number of lymphosarcomas (figs. 7–10, 12–15, and 18). This particular chromosome probably represents the Y chromosome in the lymphosarcomas originating in male mice, whereas it appears to be the result of a chromatid or chromosome deletion in the leukemic cells of female mice.

DISCUSSION

The leukemias used for the chromosome studies were induced by 1 or 2 subcutaneous injections of DMBA into newly born mice of the low-leukemic Swiss stock. Lymphosarcomas of the thymus started to develop after a relatively short induction period of 9 weeks and reached around 50 percent at 4 months after the DMBA application—an observation in agreement with an earlier report (*5*). The leukemias were of the stemcell type, with infiltrations in the lymph nodes, lung, kidney, and liver, and resembled the leukemia obtained after prolonged application of DMBA in adult mice of different strains (*11–14*). The tumor could be transplanted in some instances to adults of the same mouse strain and induced a subcutaneous lymphosarcoma on the side of the injection, an invasion of different organs,

or both. The uniform pathological appearance of these leukemias made them particularly suitable for comparative chromosome analysis.

The 16 lymphosarcomas of the thymus examined consisted of cells having an altered chromosome complement. The most persistent change was an increase in chromosome number to 41, from the normal diploid complement of 40. In addition, cells containing 42 chromosomes were present at a varying degree in most thymic lymphosarcomas. These chromosomal abnormalities are apparently associated with the process of leukemogenesis since they are either absent in normal mouse tissue or seen only in an extremely small percentage, which is generally attributed to counting errors rather than to a real variation in chromosome number (*4, 7, 15, 16*). Both these aneuploid cell lines with 41 and 42 chromosomes are in cervical, thoracic, mesenteric, and axillary lymph nodes and spleen, if these organs are infiltrated with leukemic cells. The proportion between the two cell lines might remain the same as in the thymic lymphosarcoma or might shift.

A certain regularity emerges by comparing the chromosome pattern of the 16 leukemias examined, in spite of the limitation of the cytological technique. Only cells with two aneuploid values, mainly with 41 and 42 chromosomes, are in leukemic tissues, which appears to be a very selective phenomenon taking into consideration that 39 possibilities exist for aneuploid chromosome numbers between a diploid and tetraploid chromosome complement. In addition, cells with 41 chromosomes constitute 75 percent of the aneuploid cells in all leukemias examined. The preference of one aneuploid value and the extremely small scattering of the chromosome number can hardly be the result of completely random processes, and the action of some selective mechanism must be postulated. Either the effect of DMBA on the chromosomes is selective, which appears unlikely considering the random effect of mutagens and chromosome poisons, or a particular selection occurs of originally random alterations of the chromosome complement, which is apparently true in precancerous stages of hepatogenesis due to *N,N*-dimethyl-*p*-phenylazoaniline (*17*) and during the development of malignant cells in tissue culture (*18*). Of interest in this connection are the chromosome numbers of lymphatic leukemias induced by X rays (*4*) and those of spontaneous leukemias of AKR mice (*6, 7*), which are scattered in the narrow range of 40 to 45 chromosomes with cells having 41 chromosomes occurring most frequently. The similarity in chromosome number of spontaneous, chemically induced, and radiation-induced leukemias seems to point to a common selection mechanism, since it appears unlikely that these 3 different carcinogenic factors will produce directly the same chromosome abnormalities. At present, however, little is known about the type of selection (*18*), though it appears to be organ specific (*19*).

The study presented here shows a correlation between altered chromosome complements and the occurrence of neoplastic cells, and the preference of one particular chromosome number. It is at present difficult to evaluate whether the malignant properties depend on this altered chromo-

some complement. In support of this idea the loss of malignant properties by alterations of the particular chromosome combination of Novikoff hepatoma cells can be quoted (*20*).

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PLATE 97

FIGURE 1.—Normal thymus of a 3-month-old mouse and a greatly enlarged thymus of a leukemic mouse 3 months after DMBA injection.

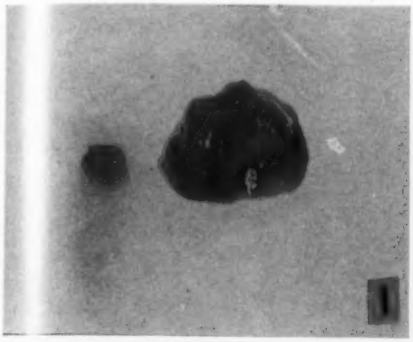
FIGURE 2.—Normal spleen of a 2-month-old mouse and greatly enlarged spleen of a leukemic mouse 65 days after DMBA injection.

FIGURE 3.—Lymphosarcoma of the thymus. Hematoxylin and eosin stain.

FIGURE 4.—Infiltrated kidney. Hematoxylin and eosin stain.

FIGURE 5.—Infiltrated lung. Hematoxylin and eosin stain.

FIGURE 6.—Infiltrated liver. Hematoxylin and eosin stain.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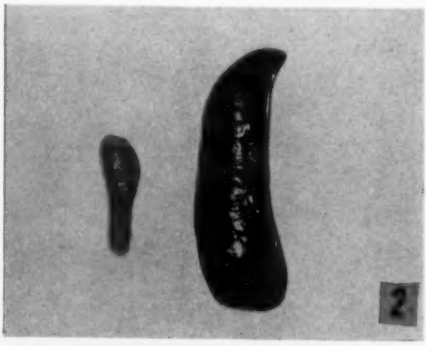
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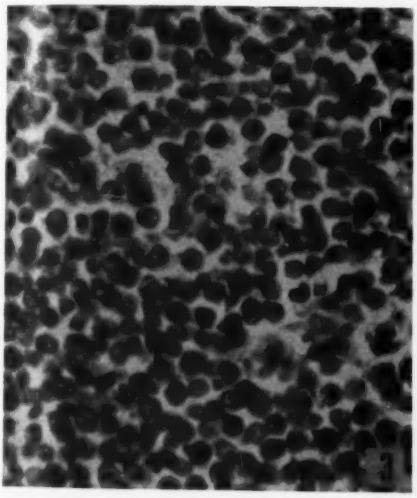
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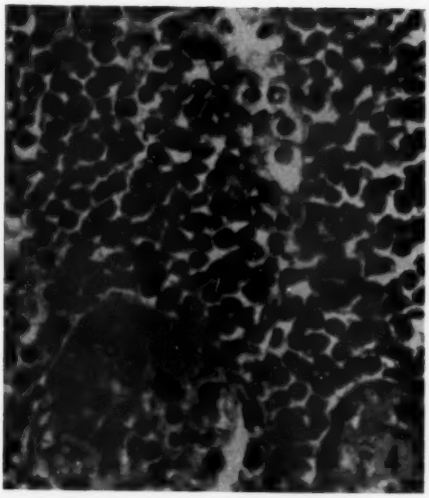
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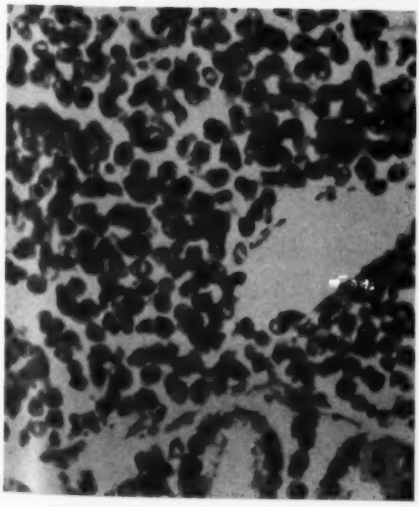


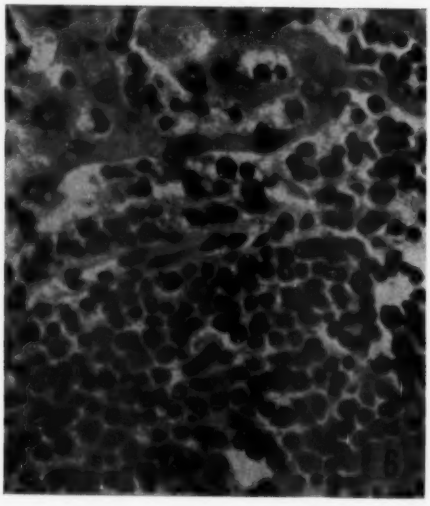


PLATE 98

Photomicrographs of orcein-HCl-stained metaphases. M = small single chromosome; S = smallest homologous chromosome pair.

FIGURE 7.—Metaphase of thymic lymphosarcoma of mouse #1 containing 41 chromosomes.

FIGURE 8.—Metaphase of thymic lymphosarcoma of mouse #2 containing 41 chromosomes.

FIGURE 9.—Metaphase of thymic lymphosarcoma of mouse #3 containing 41 chromosomes + 2 chromosomes.

FIGURE 10.—Metaphase of thymic lymphosarcoma of mouse #5 containing 41 chromosomes.

FIGURE 11.—Metaphase of thymic lymphosarcoma of mouse #8 containing 42 chromosomes.

FIGURE 12.—Metaphase of thymic lymphosarcoma of mouse #9 containing 41 chromosomes.

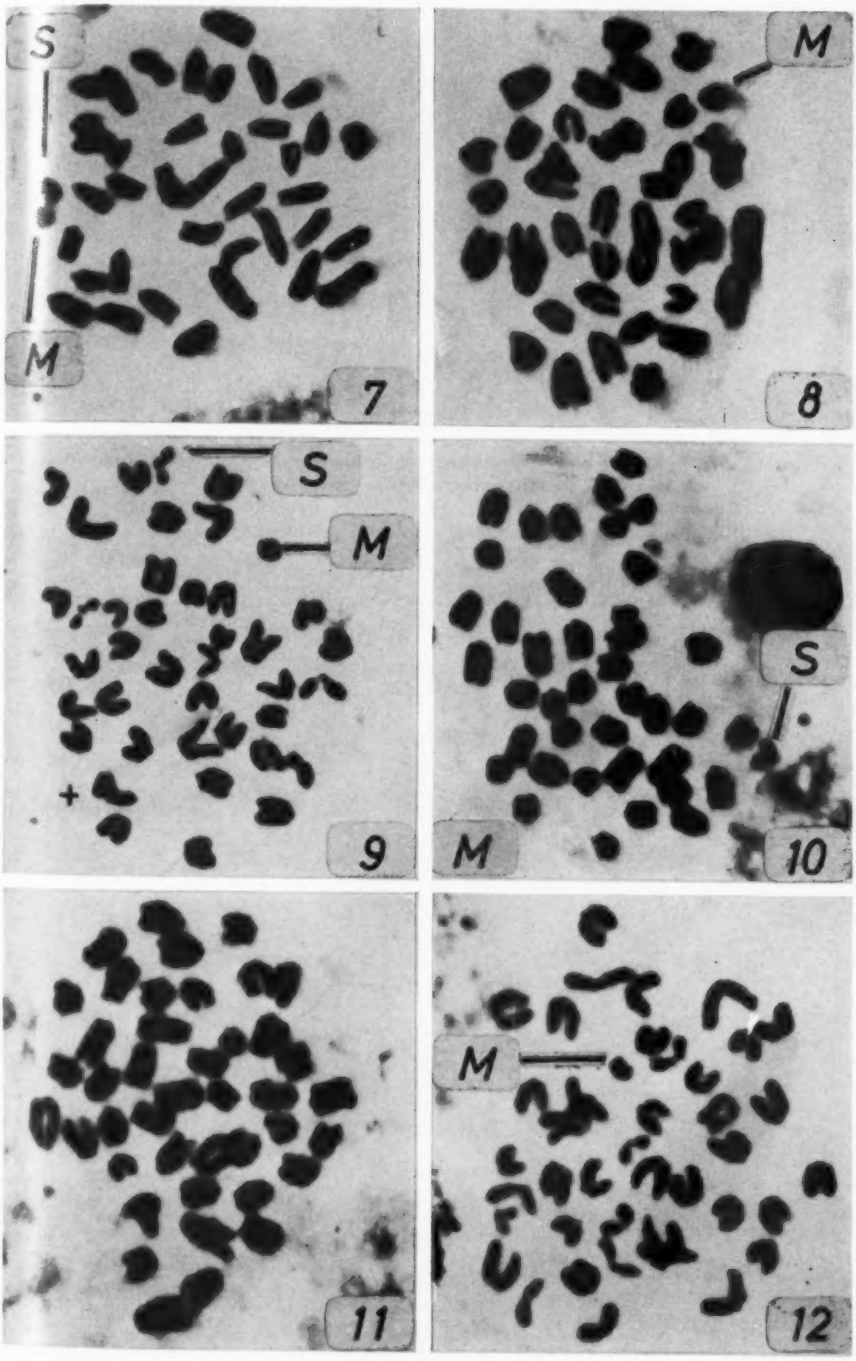

S
M
7
M
8
S
M
+
9
S
M
10
M
12

PLATE 99

Photomicrographs of orcein-HCl-stained metaphases. M = small single chromosome; S = smallest homologous chromosome pair.

FIGURE 13.—Metaphase of thymic lymphosarcoma of mouse #10 containing 41 chromosomes.

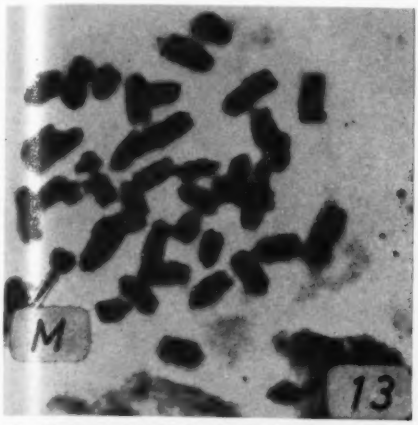
FIGURE 14.—Metaphase of thymic lymphosarcoma of mouse #11 containing 41 chromosomes.

FIGURE 15.—Metaphase of thymic lymphosarcoma of mouse #13 containing 41 chromosomes.

FIGURE 16.—Metaphase of thymic lymphosarcoma of mouse #12 containing 41 chromosomes.

FIGURE 17.—Metaphase of thymic lymphosarcoma of mouse #15 containing 41 chromosomes.

FIGURE 18.—Metaphase of thymic lymphosarcoma of mouse #16 containing 41 chromosomes.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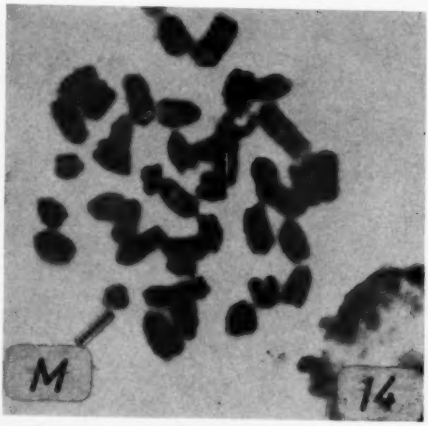
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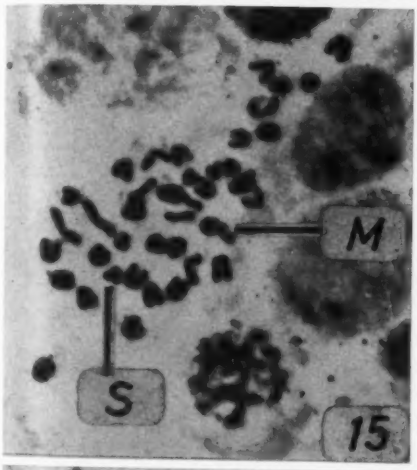
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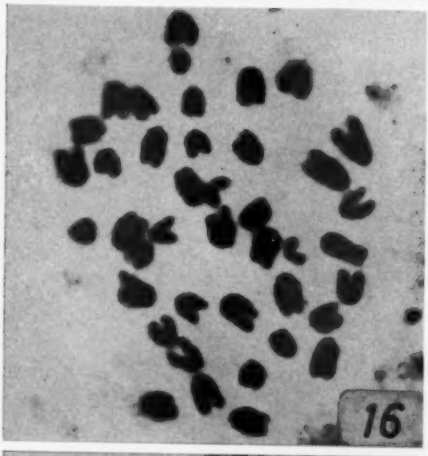
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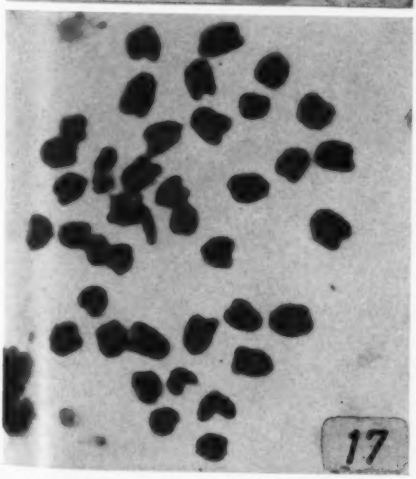
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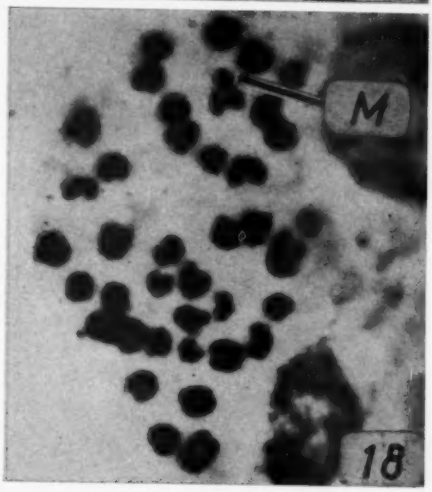
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An Explosion Instrument for Disrupting Tissues and Cells [1]

JAMES A. REYNIERS *and* MIRIAM R. SACKSTEDER,[2]
Germfree Life Research Center, Tampa, Florida

SUMMARY

A tissue gun (Model A .22 caliber) is described which uses commercially available .22 caliber blank cartridges developed for power tools such as stud drivers and a captive piston to drive tissue through screens of various pore sizes, or through matrices of quartz, carborundum, ceramics, or other abrasive materials. The same method may be used for greater volumes, with a similar apparatus of larger dimensions. With a solid disc, instead of screens, the gun can also be used to apply high pressures of short duration to liquids or cells in suspension as a means of sterilizing them. Heat, gases, and unburned powder do not reach the tissues because of the close tolerance in the construction of the piston. No frothing or heating of the tissues takes place during disintegration. While this gun was designed primarily for use in germfree isolators, it has much wider applicability in the laboratory. A particular advantage is that both the gun and the cartridges can be sterilized. The gun is made entirely of stainless steel and is sturdily constructed to contain the pressures developed with the cartridges recommended. It is simple to use and involves little time. Safety features have been built into the gun to prevent accidental discharge or to check the piston if an empty gun is fired.—J. Nat. Cancer Inst. 25: 663–681, 1960.

HIGH PRESSURES of short duration, as in an explosion, to force living tissue through disintegrating matrices or to exert force on living cells depend on a safe and practical instrument. Within the limits of its maximum rated pressure, the gun described in this report is such an instrument.[3] It was designed primarily for use inside germfree isolators but is equally useful in other types of laboratory work for a much wider range of practical applications, for example, in the selective separation

[1] Received for publication March 29, 1960.

[2] We wish to thank Mr. V. C. Stock and Mr. Lester Baker, Lobund Institute, Machine Shop, for their help in building a laboratory model for use in the germfree isolators. Our appreciation is also extended to Mr. Robert E. Reyniers, Reyniers and Son, Chicago, Ill., for making the final models of the gun. We are also grateful to Dr. R. C. Wyckoff, Washington, D.C., Dr. C. S. Cummings, Remington Arms Company, Inc., Bridgeport, Conn., and Dr. L. B. Tuckerman and Dr. J. R. Franklin, of the Bureau of Standards, Washington, D.C., for their calculations of pressures involved. We acknowledge the friendly assistance of Dr. Masasumi Miyakawa, Department of Pathology, Nagoya, Japan, Dr. Alton R. Taylor, Virus Division, Parke, Davis & Company Detroit, Mich., and the University of Notre Dame, South Bend, Ind., for facilities to test the gun in its initial development.

[3] Available from Reyniers and Son, 3806 N. Ashland Ave., Chicago 13, Ill.

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of subcellular components, for the instantaneous sterilization or inactivation of suspensions and liquids, and in studies on the physiology of cell constituents.

Tissues and cells may be broken up inside germfree isolators by conventional implements and procedures, such as electrically driven homogenizers, glass grinders, tissue presses, mortar and pestle, or freezing and thawing (*1*, *2*). The main requirement is that the apparatus can be sterilized, introduced into the isolator, and operated without becoming contaminated with bacteria. Convenience is also an important consideration. Working with a germfree isolator is restrictive with respect to space and freedom of operation. The instrument used should be small enough to pass through the sterilization locks and simple to use within the isolator.

The tissue guns described meet these requirements. One model of the gun makes use of the explosive force of gunpowder. A second model makes use of highly compressed gas. The explosion acts on a captive piston to exert high pressures of short duration on tissues or cells. In this brief interval the tissues may be forced through screens, against shearing edges, through tightly fitted plugs, or through quartz, ceramic, carborundum, and other disruptive matrices. No heat reaches the tissues from the explosion nor do gases or particles of unburned powder. The piston cannot leave the barrel since the head is larger than the exit holes.

Pressures may be regulated in steps: by gas-relief plugs in the explosion chambers, by a hollow piston which can be plugged to different volumes and so regulate the gas volume between the end of the cartridge and the head of the piston at the time of the explosion, by adjusting the powder charge, and by adjusting the length of thrust of the piston head. These adjustments are not variable, thus adding to the safety of the operation.

The volume of the tissue-loading chamber of the gun may be regulated in steps, from the upper limits of any model to a safe lower limit by volume-restricting plugs.

These guns may be made entirely of stainless steel to permit sterilization. That portion of the gun in direct contact with tissues may be fitted with a nylon or Teflon liner to avoid metal contamination, which may be desirable for such work as enzyme studies. Gunpowder cartridges and compressed nitrogen or carbon dioxide cylinders (*3*) may be sterilized if proper precautions are observed.

The guns are safe if used with loads which they are designed to accommodate. *However, they should never be fired without a full load of tissue and without the disruptive matrix in place.*

While the piston cannot leave the barrel if the gun is empty when fired, both the piston and bore will be severely damaged. It must be realized that *very high* momentary pressures are exerted; the guns must be used as intended. Also, such guns must be designed and made to contain the high pressures and should not be "home-made."

APPARATUS AND METHODS

Only one model of the tissue gun is described in detail in this report (Model 22–D). This model uses the explosive force of gun powder. A second model (Model CO–B) is mentioned only for completeness. The latter model uses the force of suddenly released nitrogen or carbon dioxide highly compressed in metal cylinders. The basic elements of design are essentially the same in all models.

Tissue Gun Model 22-D

This model of the tissue gun is intended to be used with .22 caliber blank cartridges developed by the Remington Arms Company, Inc., Bridgeport, Connecticut, for its Remington Stud Driver. The cartridges are available commercially in many different loadings. For all but a few experiments, the *yellow* load, containing about 156.6 mg. of powder, is satisfactory. The weaker *green* load, containing 128.2 mg. of powder, is equally useful. In a few instances, especially with plug matrices, the *red* load, with 182.6 mg. of powder, has been used. In principle, the weakest load should be used first in all experiments until a higher-power load is indicated.

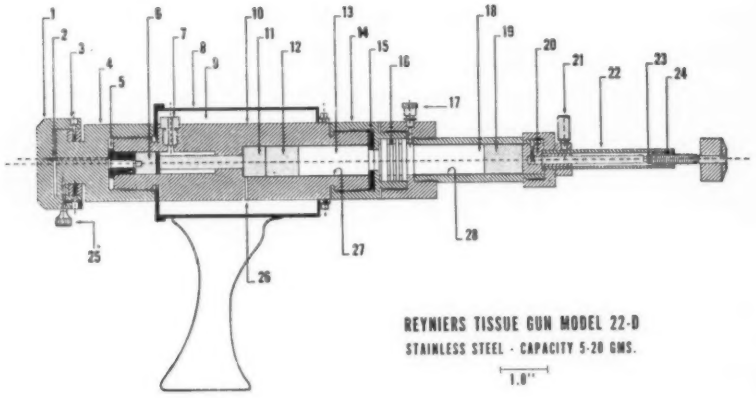
Text-figure 1 illustrates the construction of the gun which is made entirely of stainless steel (Allegheny metal 410–TB12). The assembled gun is shown in figure 1 and parts are shown in figure 2. Pressures are controlled by the following factors: (*a*) weight of powder loaded; (*b*) insert plugs with openings of different diameters, which are screwed into the explosion chamber; (*c*) a hollow piston, in which the volume can be varied by plugs, thus permitting variation in the distance between the end of the cartridge and the space in the piston and expansion of powder gas before the piston is moved. Variations in pressure will depend on the type of tissue loaded, also, the type of disruptive matrix, and the number of volume-restricting plugs inserted in the tissue chamber (text-fig. 2).

The maximum capacity of the gun is about 22 gm. of tissue, which can be reduced, by decrements of 5 gm., to a minimum of 5 gm. by the use of restrictive plugs. However, since a certain amount of tissue is lost between the screens and in the space before the screens, a safe operating minimum is 10 gm. of tissue.

Tissue-disruptive matrices

These are all of the same diameter to fit the matrix chamber in the Model 22-D gun. A wide variety of matrices may be used to break up tissues or cells and to suspend or separate the homogenates. Four general types are described: screens, plugs, cells, and tubes; they are illustrated in text-figure 3.

Screens (*text-fig. 3A*).—These screens vary in thickness and in the number and diameter of the holes. They may be used singly or in combinations. In all instances the screens are separated by nylon spacer



TEXT-FIGURE 1.—Sectional view:

1—Breech cap
2—Firing pin
3—Retaining screw
4—Breech block
5—Cartridge holder
6—No. 1 piston
7—No. 4 gas relief plug
8—Filter muffler
9—Glass fiber
10—Barrel
11—S. S. tissue plug
12—No. 1 nylon plug
13—Tissue space
14—Muzzle nut A
15—Safety anvil
16—Space for tissue screens
17—Plug 10 gauge hypo. needle
18—Tissue collection space
19—Volume restrictor plugs
20—Pusher head
21—Set screw
22—Push rod
23—Vernier screw
24—Vernier nut
25—Safety pin
26—Air vent to seat piston
27—Nylon liner optional
28—Nylon liner optional

rings. They are held firmly in place by pressure from the muzzle nut. When the screens are used in combination, the holes in each screen do not line up, so that tissue is forced through the screens in zigzag fashion, to impart a shearing action. The space between the screens formed by the spacer rings further enhances this action.

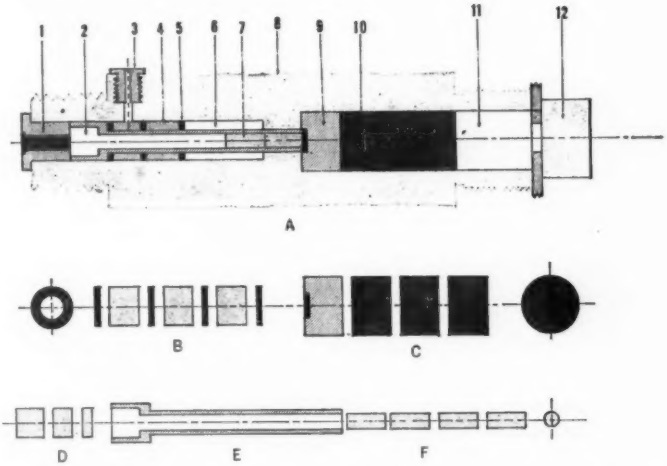
There are two types of screens: DSA, which is .125 inch thick and is made of stainless steel hardened to Rockwell 50, and DSB, which is .0265 inch thick and is made of perforated metal plate. The screens are circular and have the following diameters: DSA—.750 inch, DSB—1 inch. The number and diameter of the holes for each screen are given as fractions, in which the numerator represents the number and the denominator, the diameter, in fractions of an inch. Standard screens used in this report are: DSA 60/16, DSA 170/32, DSA 379/.024, DSB 714/.020 s (staggered) or r (regular); DSB 952/.020 r; DSB 517/.024 r.

The spacer rings are all 1 inch in outer diameter by .750 inch in inner diameter but vary in length from .625 inch to .500 inch by increments of .0625 inch. They are made of nylon, Teflon, or stainless steel and are used to separate screens or to make cells.

Cells (*text-fig. 3B*).—These are usually made of a combination of screens and spacer rings. The space between the screens is filled with abrasive

materials, such as carborundum, quartz, stainless-steel shavings, ground glass, sand, ceramics, and/or fibers such as asbestos and sintered (porous) steel. While the cells, in combination with screens, may be used with whole tissue, they are usually used with cell suspensions or tissue which has been homogenized, *i.e.*, passed once through the gun.

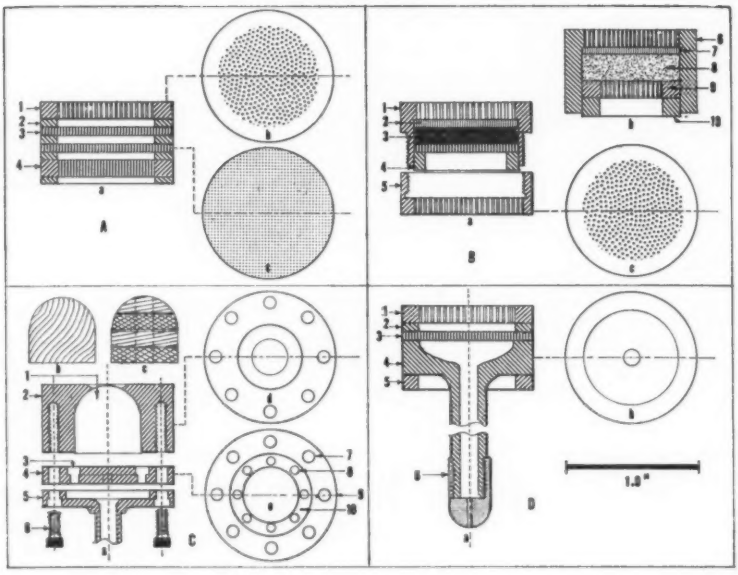
Plugs (*text-fig. 3C*).—The plugs are made from round files, burrs, smoothly tapered plugs, and ceramic grinding wheels of different diameters



TEXT-FIGURE 2.—Schematic representation of explosion-pressure control, tissue volume restriction, and piston retention in tissue gun Model 22–D. *A.* Assembled section of tissue gun: (1) cartridge holder; (2) head space in piston; (3) gas-pressure relief plug; (4) piston-shaft spacer rings for restricting length of thrust; (5) nylon spacer ring; (6) explosion chamber; (7) piston-shaft plugs for restricting thrust of piston; (8) barrel of gun; (9) stainless-steel tissue plug; (10) nylon or Teflon plugs for restricting volume of tissue chamber; (11) tissue chamber; (12) safety anvil, which is screwed into muzzle nut and prevents the tissue-restricting plugs from leaving gun. *B.* Piston spacer rings, used to restrict the length of travel of the piston, which should correspond to available length of the tissue chamber; the spacer rings between the steel spacers are nylon. *C.* Tissue-restricting plugs; plug in contact with the end of piston is stainless steel; other restricting plugs are nylon or Teflon, .500-inch long, and each decreases the volume by about 5 cm.^{3} *D.* Plugs are different lengths which fit into the head of the piston and restrict volume of gas expansion after explosion. *E.* Hollow piston. *F.* Plugs for piston shaft; can be made to any length; those shown are .500-inch long and will fill completely the shaft of the piston or any portion of it.

and shapes. They are accurately fitted into a die and clamped tightly by six Allen-head screws. The tissue is forced between the die and the ram or plug. Smooth plugs are used with tissue homogenates in suspension, to separate and size the tissue particulates.

Tubes (*text-fig. 3D*).—These tubes may be used alone or in combination with screens and spacer rings. The length and number of the tubes and the diameter or shape of the holes may be varied. These tubes are used



TEXT-FIGURE 3.—Tissue-disruptive matrices (only general types are shown). Each may be used in many combinations. All are made to fit the space in the muzzle nut of the gun. *A. Screens:* (1) DSA screen .125-inch thick with different number and diameter of holes; (2) nylon spacer ring used between screens to allow tissue to spread out before it is pushed through next matrix; (3) DSB screen made from perforated metal plate; (4) DSA screen—usually with smaller diameter and greater number of holes; (b) top view of DSA screen; (c) top view of DSB screen. *B. Cells:* (a) sectional view of model 1 tissue cell; (1) DSA screen; (2) DSB screen; (3) asbestos or stainless-steel shaving pad compressed by threaded cap; (4) spacer ring—must be long enough to permit compression of pad; (5) threaded cap with perforated top; (b) Model 2 type cell; compression of the fill is obtained from muzzle nut; (6) perforated stainless-steel cell casing; (7) DSB screen with perforations small enough to contain powdered or fragmented fill; (8) fill of carborundum, quartz, ceramics, etc.; (9) DSA screen; (10) spacer ring long enough to permit compression of fill; (c) top view of cell. *C. Plugs:* Tissue is pushed between tightly fitted surfaces which can be smooth or rough; (a) sectional view of plug housing; (1) this space closely fitted to conformation of plug and may be any shape; plugs may be round files, burrs, or grinding wheels; (2) stainless-steel die housing; (3) annular ring into which homogenized tissue is pressed; (4) plate; (5) collecting plate and tube; (6) Allen-head cap screws for putting pressure on plug; (b) round file; (c) type of plug made from sections of screw threads and burrs; (d) bottom view of die housing; (e) top view of annular-ring plate; (7) holes for cap screws; (8) holes in annular ring through which homogenized tissue is passed; (9) plate made of stainless-steel hardened to Rockwell 60; (10) annular ring. *D. Tubes:* Generally used in combination with screens to suspend and further separate particulates from tissue homogenate. (A) section; (1) DSA screen; (2) nylon spacer ring; (3) DSB screen; (4) homogenizing tube; (5) nylon spacer ring; (6) threaded cap with either single or multiple holes of different diameters; (b) top view of homogenizing tube.

primarily for making suspensions of homogenized tissue in a diluent contained in the tissue-collecting tube.

Pressure control (text-fig. 2).—Explosion pressure is dependent upon the weight of powder or the powder charge. It may be further controlled by the use of pressure-relief plugs screwed into the explosion chamber and placed ahead of the piston when it is seated. These plugs increase in size by increments of .031 inch from a blank to .125 inch. The pressure is also controlled by the use of gas expansion chambers located in the head of the piston. These vary in depth from a blank to .375 inch by increments of .031 inch. Maximum power for any load is obtained by using a blank gas-relief plug and a solid piston head.

Short of actual physical measurement, it is difficult to do more than estimate the pressures exerted on the tissues and cells with different power loads (*4–6*). The exact effect of momentary high pressures on tissues and cells is unknown and largely a matter of estimation and experiment. The following equation for estimating explosion pressure is offered as a guideline for these experiments: [4]

$$Pe = \frac{F}{\frac{1-A}{D}}$$

Pe [1] = peak explosion pressure, in psi;
F = powder force, in psi × cc. per gm.;
D = loading density, *i.e.*, volume available to powder gases, in gm. per cc.;
A = covolume of powder gases (0.925).

For the powders used take F = 140,000 psi.
F will be a decreasing function of time due to heat loss.
The peak of the explosion is less than a millisecond.
The initial volume VI available to the powder gases in Model A .22 caliber tissue gun varies with the piston head used.

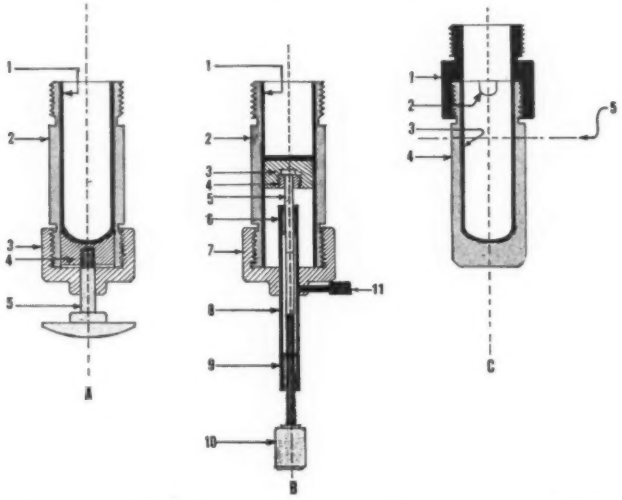
Finally, the pressure developed within the gun is also affected by the weight of the tissue load, the density of the tissue, and the kind of matrix through which the tissue is forced. Only theoretical estimates of these factors can be made at present.

Safety control.—Working with pressures as high as those used in the tissue gun requires assurance that the design and construction of the gun is adequate. The gun is constructed of stainless steel (Allegheny Metal 410, Type 12TB), which is a high-impact alloy. The gun has no trigger and can be discharged only when a hardened pin is pulled completely free of the breech cap, after which the cap must be struck. The piston head is greater in diameter than the bore, as is the plug which is separated from the piston. This prevents either from leaving the gun. The piston head is backed with a nylon washer; the plug strikes against a hardened anvil and is further cushioned by a nylon plug, which restricts the space. The barrel of the gun is quite massive and the screw threads for the breech

[4] These calculations were arrived at independently by Dr. C. S. Cummings, Remington Arms Company, Inc., Bridgeport, Conn., and Dr. R. C. Wyckoff, Washington, D.C.

cap are very heavy to withstand the shock. In short, all the safety features required have been built into the gun. Nevertheless, the gun should not be handled carelessly, with stronger loads than recommended, or discharged unless fully loaded with tissue. If it is used with 10 gm. or less (5 gm. minimum) of tissue, only the weakest (*green*) load should be used.

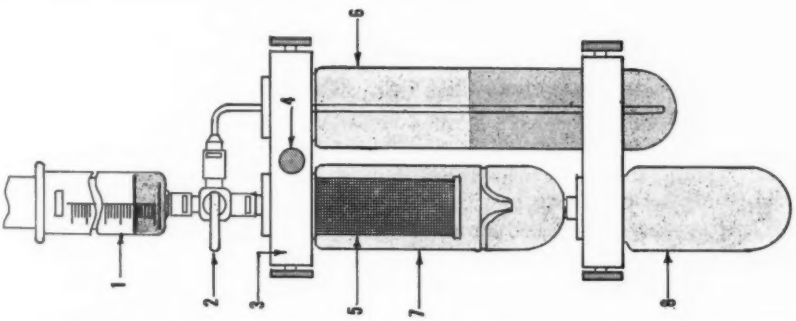
Restrictive plugs and liners.—The volume of the tissue chamber may be restricted by the use of nylon or Teflon plugs. These are .500 inch long and fit the bore closely so that for each additional plug the weight of tissue is decreased by about 5 gm. For each added plug, a corresponding sleeve of stainless steel may be placed on the shaft of the piston. In experiments where contact between the tissue and the stainless-steel bore is objectionable, as in some enzyme assays, nylon or Teflon liners may be used. These fit the bore snugly and have a wall thickness of .0625 inch. They must be used with the proper diameter piston and plugs.



TEXT-FIGURE 4.—Tissue-collecting tubes. (A) Sectional view of standard collecting tube, which can be used with plastic liner. (1) Plastic liner—must fit closely to walls and bottom of tube because of force of expressed tissue; (2) stainless-steel collecting tube, which fits into muzzle nut; (3) cap with plug to push plastic liner; (4) plug; (5) push rod. (B) vernier tissue-collecting tube—standard tube fitted with a vernier cap, made to push homogenized tissue into attached hypodermic syringe to eliminate weighing and possible contamination; (1) plastic liner; (2) standard tissue tube; (3) plug; (4) nut for holding end of push rod to permit it to turn independently of plug; (5) screw; (6) plug to guide threaded rod; (7) cap; (8) coarse-adjustment push rod; (9) threaded nut; (10) knurled screw head; (11) setscrew—tightens on coarse adjustment push rod, after which screw may be used for fine adjustment. (C) freezing tube, used to freeze homogenized tissue immediately. (1) plastic nut, nylon or Teflon, used to insulate tube from barrel of gun; (2) cutout in collecting tube to permit removal of plastic liner; (3) plastic liner; (4) stainless-steel collecting tube; (5) immersion line. Tube is immersed in liquid air or alcohol-dry ice bath.

Tissue-collecting tubes (*text-fig. 4*).—These tubes in which the homogenized tissue paste is collected screw into the muzzle nut. There are three general types in use: standard (text-fig. 4A), vernier (text-fig. 4B), quick-freeze (text-fig. 4C). The standard collecting tube is stainless steel, has a screw cap, and may be used with a plastic centrifuge tube that fits snugly. If a plastic liner is used, a vernier cap is useful to push the liner from the collecting tube. The vernier cap is screwed onto a standard collecting tube and consists of a push rod, a set screw to fix the push rod after the tissue is pushed into place, and a screw to measure accurately the amount of tissue pushed into the attached syringe. The quick-freeze collecting tube is a standard tube screwed into a plastic bushing; the latter is necessary to insulate the portion of the collecting tube in contact with a low-temperature bath from the main body of the gun.

Tissue-suspension tube (*text-fig. 5*).—If a vernier collecting tube is used, the tissue paste may be delivered into an attached syringe to any desired amount. The syringe is then removed from the gun and attached to a hypodermic needle, and the desired amount of fluid is pulled into the syringe and flushed several times until suspension is accomplished. The syringe is then attached to a tube containing a double fine-mesh-screen cylinder, such as is used in blood transfusions, through which the suspension is strained; or, a filter may be substituted for the screen, thus effecting filtration immediately. The point is that with a vernier cap it is unnecessary to remove the tissue from the gun by disassembling it, so thereby permitting effective control of contamination.



TEXT-FIGURE 5.—Tissue suspension bottle. After homogenized tissue is forced into syringe attached to gun, syringe is fastened to tissue suspension bottle. Diluent is drawn into syringe and the tissue suspended by flushing, after which the suspension is passed through double stainless-steel screens or a filter. (1) Syringe containing homogenized tissue; (2) three-way valve; (3) yoke for holding bottles; (4) rod for attaching to stand; (5) double stainless-steel fine-mesh cylinder; (6) bottle containing the diluent; (7) blood drip tube; (8) bottle for receiving filtered tissue suspension.

Tissue Gun Model CO-B

This gun, shown in figure 3, is operated by sudden release of a gas (carbon dioxide or nitrogen) from small cylinder under high pressure.

It is a commercially available gas-operated pistol, which has been adapted to suit our requirements.[5] The tissue barrel, screens, and collecting tube are of stainless steel, but the rest of the pistol is not, so that it cannot be sterilized with heat or steam. Amounts of tissue from 0.5 to 3 gm. may be accommodated. The pressures exerted are not as high as with gun powder, but the design and the principles involved are the same.

Use of Model 22-D Tissue Gun

At the present time experience alone determines the best combination of loads, disruptive matrices, pressures, and preparation for any particular tissue. The following procedure utilized tumor tissue (chicken fibrosarcoma) in making serial passages and in working with fractions of the tissue. Since the gun was used in a germfree isolator, sterilization of the gun components and cartridges was necessary. In normal laboratory use such sterilization may not be required.

Steps in Using a Tissue Gun Inside Germfree Isolators

1. The gun is disassembled and washed in detergent with brushes or gun-cleaning implements. The parts are then rinsed and dried with lintless cloth. Powder traces on the end of the piston and in the explosion chamber may be removed with a powder solvent and a gun-cleaning brush. Polishing cloth should be avoided unless it is used with care because of the danger of altering the bore and destroying the fit between the piston and bore.

2. The parts may be sterilized by autoclaving at 252° F. for 25 minutes, drying for 25 minutes at 28-inch Hg vacuum, and then moved into a germfree isolator through a sterile lock. The cartridges may be sterilized at the same time by standing them upright on their bases in a small pan with a fine-mesh screen bottom. The cartridges are placed near a light bulb inside the isolator for additional drying. We have never had a cartridge explosion during sterilization, and few have failed to discharge. However, should such an explosion take place no damage would ensue. Since the cartridges are blanks, they are in the sterile lock, and not closely confined and only a small number (4 to 6) are sterilized at one time.

3. Prior to use of the gun the piston, piston sleeves, and screens are selected, and the gas-relief check is screwed into place. The gun is then assembled and checked to be sure that the piston, tissue screens, and restricting plugs are firmly seated. The desired disruptive matrix is assembled in the split muzzle nut and the tissue-collecting tube screwed into place. The cartridge is placed in the gun after tissue is loaded, just before it is discharged.

4. Tissue is loaded into the gun, cut into small pieces with scissors, and lightly packed into place with a ram to make a firm pack that fills the entire space.

5. The muzzle nut is now screwed onto the gun barrel.

[5] Crosman Model 157 gas-operated pistol, Crosman Arms Co., Inc., Fairport, New York.

6. The proper power load is selected and packed into the cartridge holder, which is then pushed into the piston end of the barrel. After the safety pin is pushed in, the breech block is screwed firmly into place.

7. The muffler and filter are packed prior to sterilization with FG-50, AAF glass-fiber filter mat and fastened onto the barrel of the gun. The gun is then ready to be discharged.

8. The safety pin is pulled out of the firing cap as far as it will go and the cap is tapped lightly against the underside of a surface (so that the gun is discharged pointing at the floor) or tapped with a plastic-head mallet. Depending on the aperture of the gas-relief check, the sound of the explosion will vary from none with a solid plug to a light, sharp crack.

9. The tissue that passes through the disruptive matrix will enter the collection tube as a very smooth paste. It is unnecessary to dilute the tissue prior to firing to break it up, but this can be done if desired. After homogenization, the tissue may be removed from the collecting tube and suspended in a diluent, or a vernier cap may be attached to the collecting tube and the tissue paste forced out into an attached syringe in which it can be further suspended. This latter step is important for operations within a germfree isolator, since it eliminates the need to weigh the tissue and suspension is made easier.

10. After each use the gun should be disassembled and cleaned.

Special Uses of the Tissue Gun

The gun can be adapted to many problems involving the effect of momentary high pressures on living tissue or cells, their breakdown, and the selection of particulates. A few of the special uses are now mentioned.

Tissue may be frozen immediately after homogenization, a procedure of value in enzyme-antienzyme studies, in the following manner: The use of an insulating nut screwed into the muzzle cap and into which a collecting tube is screwed permits immersion of the collecting tube in liquid air or an alcohol-dry ice mixture. The insulating nut prevents the gun from freezing. Cells in suspension, *e.g.*, yeast, bacteria, or tissue may be subjected to high pressure of extremely short duration with the use of a solid plate in the matrix chamber. Traces of gas or chemicals can be incorporated in the reaction. Such experiments are of value in studies involving cell injury, nuclear damage, or instantaneous inactivation of enzymes.

The use of closely fitted surfaces, both smooth and rough (text-fig. 3B), through which homogenized tissue, either in suspension or unsuspended, may be forced presents interesting possibilities which need further investigation. It seems possible to effect a kind of selection of particulates by a process which is a cross between filtration and centrifugation. The use of descriptive matrices (text-fig. 3C), packed by placing under pressure fills of different thickness and particle size, such as ceramics, quartz, carborundum, or asbestos, through which the homogenate is passed, may further expand the number of potential uses.

Homogenizing tubes or nozzles are used with suspensions of tissue cells or microbial cells, and utilize the shearing action of the nozzle orifice as well as the suspending medium in the collecting tube.

RESULTS AND DISCUSSION

Use of gun in germfree isolators with chicken fibrosarcoma.—Originally, the gun was intended for use within germfree isolators to break up tumor tissue (fibrosarcoma) into whole cells or cell fractions for transplantation in animals. We wished to determine whether this could be accomplished without introducing contamination or destroying the tumor-causing properties of the homogenates. Results obtained with the tissue gun follow.

The fibrosarcoma was induced in germfree chickens by a single 0.25 ml. injection of 1.9 percent methylcholanthrene in benzene (MCA/bz)

Table 1.—Results of inoculation of 0.25 ml. chicken fibrosarcoma homogenate* prepared with gun Model 22-B and *red* power load

Experiment No.	Serum combination†	Site of inoculation	Number of takes / Number inoculated	Contamination
1	ACAR3	Breast muscle	2/2	Negative
2	AAR3	"	2/2	"
3	ACAR3	"	4/4	"
4	ACAR3	"	4/4	"
5	ACAR3	Intraperitoneal	5/5	"
6	ACAR3	"	3/3	"
7	ACAR3	"	4/4	"
8	AAR3	Breast muscle	1/2	"
9	AAR3	"	2/2	"
10	ACAR3	Intraperitoneal	3/3	"

*Supernatant, top portion of homogenate, after settling and screening, was used for all inoculations. For experiments 6 through 10, the following fritted-glass filters were used: Expts. 6-8: F = 4–5.5 mμ; expt. 9: C = 40–60 mμ; expt. 10: UF = .9–1.4 mμ.

†ACAR3 = DSA 60/16; DSB 324; DSA 170/32; SR 375.
AAR3 = DSA 60/16; DSA 170/32; SR 375.

into the right breast muscle. Whereas with conventional chickens [6] MCA/bz-induced tumors could not be transmitted to other conventional chickens by injections of cells or tissue implants, from germfree birds they were readily transmitted to both germfree or conventional birds for at least 14 passages [the number completed at present (*1*, *2*, *7*)].

In the experiments in which the gun was used, the tumor tissue was minced with scissors prior to loading. A *red* load was used for firing through the screen-matrix combinations mentioned in table 1. In more recent experiments, a *green* or *yellow* load was found satisfactory, and is preferred to the more powerful *red* load.

The homogenate was made into a 10 percent suspension in buffered saline and allowed to settle. The supernatant was injected in 0.25 ml.

[6] "Conventional" designates chickens raised under ordinary, non-germfree, laboratory conditions.

amounts into the right breast muscle or intraperitoneally. In these experiments the tumors were in the eighth to twelfth serial passage. In ten successive experiments with the tissue gun inside the germfree isolators (table 1) no contamination resulted, and there was no interference with the ability of the tumor suspensions to produce tumors on inoculation into new hosts.

It is difficult to make a comparison between the gun and an electrically driven homogenizer, *e.g.*, the Servall Omni-Mixer, in which the tissue must be homogenized with a diluent. As might be expected the Omni-Mixer produced considerable foam by mixing air with the suspension. The tissue gun, besides being simple to use, does not cause foaming or heating of the suspension during homogenization. Particulates, at two magnifications, of homogenized chicken fibrosarcoma tissue prepared with a gun are shown in figures 4A and 4B. An electron micrograph of a 20,000 rpm concentrate from a gun homogenate of monkey-embryo kidney cells is shown in figure 5.

The gun satisfies the purpose for which it was originally designed and has also proved its usefulness for other purposes in the laboratory. Both capacity and pressure of the gun may be increased by appropriate changes in design. The same is true for smaller capacities as shown in figure 3, Model CO-B.

The following observations may serve as a guide for those interested in use of the gun for homogenization of tissues and cells. With a *green* load, a constant screen combination, *e.g.*, DSA 60/16; nylon spacer; DSB 324/.020; nylon spacer; DSA 170/32; Sp .375, and the same quantity and type of tissue, *e.g.*, 20 gm. of chicken fibrosarcoma, the amount of tissue recovered as homogenate will be about 60 percent. The small amount of tissue not recovered as homogenate is lost between the screens or in the space before the screens.

The following tissues and cells have been homogenized in the gun, with the above-mentioned screen-matrix combination and a *green* load: liver, kidney, brain, muscle, skin, intestine, various tumor tissues, spleen, whole small mammals (rats and mice), chick embryos, and tissues in culture. Suspensions of yeast and bacterial cells require a different disruptive matrix and a higher *red* load. The best matrix for this purpose seems to be a cell-screen combination: DSA 170/32; nylon spacer; DSB 520/.020; nylon spacer; carborundum; DSB 520/.020. Details on microbial suspensions must await more work.

Knowledge concerning conditions involving the use of a tissue gun will remain empirical until more is known about the forces used and their effects on various tissues. In the gun described, an instrument is available that satisfies the immediate purpose for which it was made. Its convenience justifies further exploration of its potential usefulness for other purposes in the processing of biological materials.

The exact effect of pressures on the development of cells and on fractionation of the cells in connection with liberation of viruses and enzymes will be the subject of future investigations.

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(6) Corner, J.: Theory of Interior Ballistics of Guns. John Wiley & Sons, Inc., 1950.

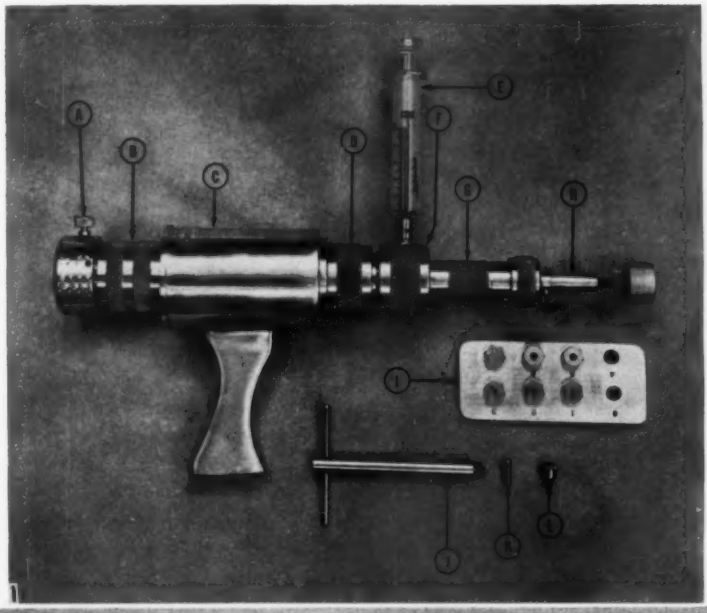
(7) Taylor, A. R., Brandon, F. B., and Reyniers J. A.: Fractionation studies of tumor tissues from germfree chickens. Ann. New York Acad. Sc. 78: 354–370 (figs. 1 and 2), 1959.

PLATES

PLATE 100

FIGURE 1.—Assembled tissue gun Model 22–D: (A) firing pin safety; (B) breech block; (C) muffler-filter-handle combination; (D) muzzle nut, containing safety anvil and chamber for tissue disruptive matrices; (E) hypodermic syringe—attached only after gun has been discharged; prior to discharging gun this opening is sealed with a plug; (F) muzzle nut containing tissue-collecting tube; (G) standard tissue-collecting tube; (H) vernier screw cap for pushing homogenized tissue paste into syringe; (I) plate containing series of gas-pressure relief plugs; (J) wrench for recovering piston after discharge of gun; (K) .22 caliber blank cartridge, used with Remington Stud Driver; (L) plug for hypodermic needle in muzzle nut.

FIGURE 2.—Disassembled tissue gun Model 22–D: (A) vernier cap; (B) standard collecting tube; (C) plastic liner, which is optional; (D) hypodermic syringe; (E) plug for hypodermic needle; (F) muzzle nut into which tissue-collecting tube is screwed; (G) muzzle nut containing safety anvil and tissue-disrupting matrix chamber; (H) muffler-filter-handle combination; (I) gas-pressure relief plug for controlling explosion pressure; (J) cartridge holder; (K) .22 caliber blank cartridge; (L) breech block; (M) spacer ring; (N) tissue-disrupting screen DSA 170/32; (O) nylon spacer ring placed between screws to permit tissue to spread before passing through next screen; (P) DSB 52/.020 tissue screen; (Q) DSA 70/16 tissue screen with nylon spacer ring; (R) safety anvil screwed into muzzle nut, which prevents tissue plug from leaving gun barrel; (S) nylon tissue-restricting plug; (T) stainless-steel tissue plug, which fits next to piston shaft; (U) nylon ring placed on piston shaft as a seal and for shock protection; (V) standard piston of stainless steel; (W) wrench for recovering piston after gun has been discharged; (X) plate containing a series of gas-pressure relief plugs; (Y) wrench for removing and tightening gas-pressure relief plugs in barrel of gun; (Z) firing safety pin. Gun cannot be discharged until pin is pulled out and breech cap is struck.



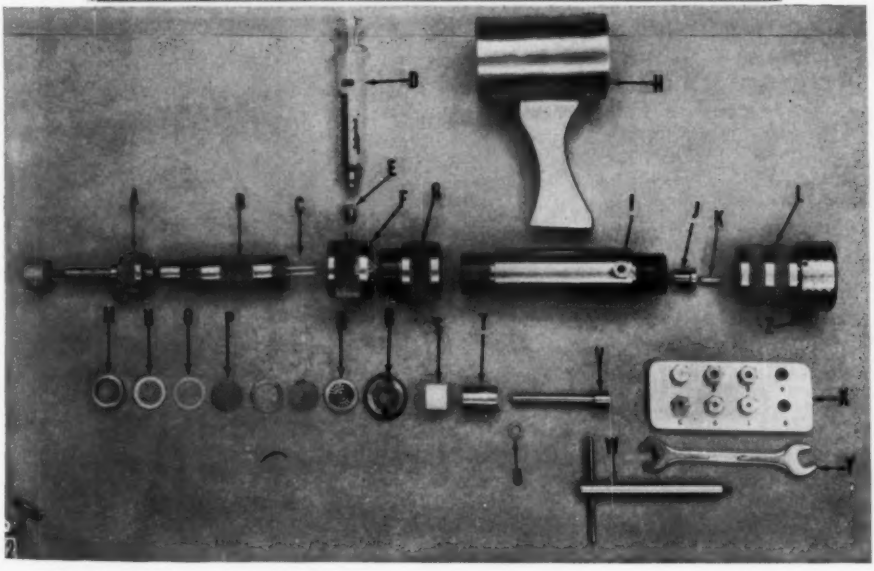


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PLATE 101

FIGURE 3.—Tissue gun Model CO-B. This model operates on compressed gas released suddenly as an explosion. It is made on a Crosman "157" gas pistol. The body of the gun cannot be sterilized but the tissue attachment is made of stainless steel and can be sterilized. (A) tissue collector; (B) tissue-disruptive matrix space; (C) tissue barrel of gun; (D) split muzzle nut to permit cleaning of barrel and access to piston so that it can be returned to barrel; (E) muzzle nut fastened permanently to barrel end; (F) barrel of gun modified to accept a piston; (G) cocking device; (H) gas-cylinder barrel; (I) gas cylinder, which can be loaded with either CO_2 or nitrogen.

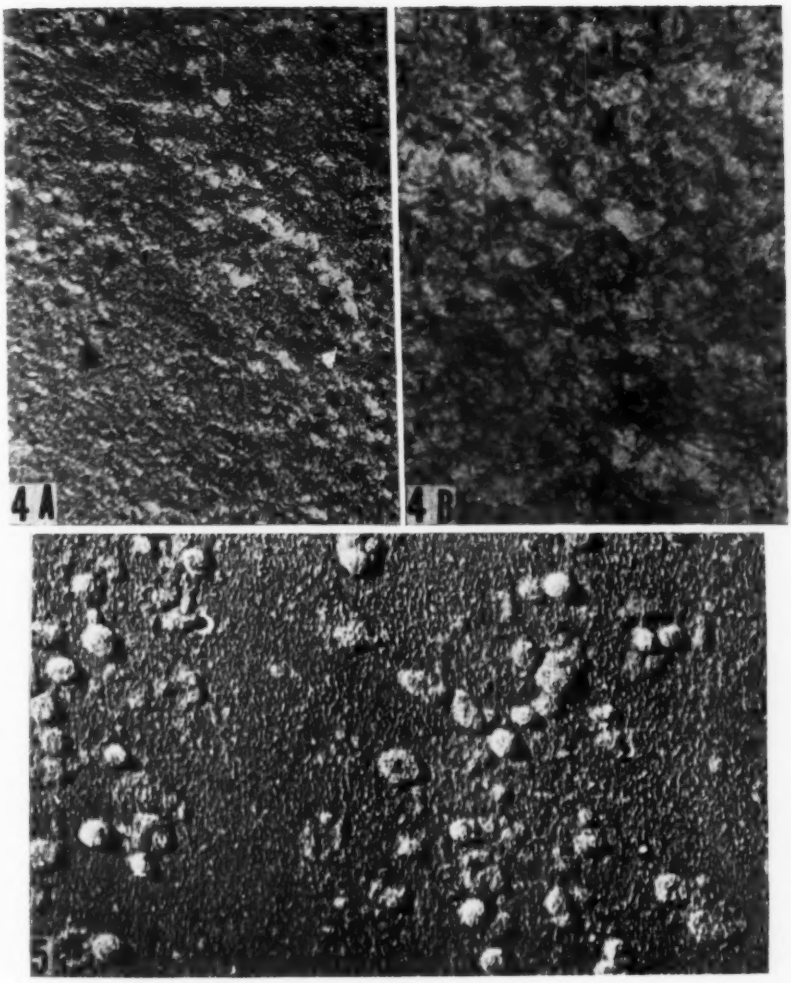
B
C
D
E
F
H
I

PLATE 102

FIGURES 4A AND 4B.—Photomicrographs of chicken fibrosarcoma passed through tissue gun Model 22-D. The conditions were: *yellow* load; standard blank piston, no gas relief, 20 gm. tissue load, screen matrix DSA 72/16; DSB 520/.24; DSA 170/32, standard collecting tube. Hematoxylin and eosin. (A) × 450; (B) × 900

FIGURE 5.—Electron micrograph of gun homogenate of monkey kidney cells; suspension centrifuged at 20,000 rpm. × 50,000

Homogenate and photograph
supplied by Dr. Alton Taylor, Parke, Davis & Co.


4 A
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Announcements

Manuscripts Approved for JNCI, July 15 to August 15, 1960

"A study of methods for abbreviating carcinogenicity bioassays. I. Enhancement of the neoplastic response by pretreating with a potent carcinogen," by William E. Poel.

"Clues to the etiology, pathology, and therapy of cancer provided by analogies with 'runt disease,'" by Albert Tyler.

"The importance of the infecting dose on the growth patterns of Rous sarcoma virus (RSV) in chick brain," by Frank J. Rauscher and Vincent Groupé.

"Heated fats as carcinogens. A critical review of experimental results," by E. Arffmann.

"Synthetic auxins in the chemotherapy of transplanted and spontaneous mouse adenocarcinoma," by C. A. Apffel, A. Tregier, and F. Homburger.

"Endometrial sarcomas of the uterus and carcinosarcomas of the submaxillary salivary gland in castrated AXC strain female rats receiving 2-diacetylaminofluorene and norethandrolone," by Melvin D. Reuber.

"Evaluation of drug resistance in cell cultures by differential toxicity tests," by Morgan Harris.

"Analysis of enzymatic activities of clones derived from variant cell lines transformed to malignant cells in tissue culture," by D. B. McNair Scott, A. M. Pakoskey, and K. K. Sanford.

"Serum protein changes in malignant disease. II. The chronic leukemias, Hodgkin's disease, and malignant melanoma," by D. R. Boggs and J. L. Fahey.

"Prolonged survival of skin homografts in parous female mice," by E. J. Breyere and M. K. Barrett.

"Mammalian chromosomes *in vitro*. XIII. Cyclic and directional changes of population structure," by T. C. Hsu.

"Growth inhibition of chick-embryo cells by some 5-fluorinated pyrimidines. Effect on Rous sarcoma virus infection," by Marvin A. Rich, Elliott H. Stonehill, and Maxwell L. Eidinoff.

"Homologous and heterologous skin transplantation in patients with lymphomatous disease," by D. G. Miller.

VIII International Cancer Congress

Representatives of the International Union Against Cancer, National Organizing Committee of the U.S.S.R., met in Moscow, in May 1960. The conference resulted in the following suggestions to be presented for final action by the Executive Committee, which will meet in Tokyo, October 1960:

Date of Congress: July 23 to 28, 1962.
Sixteen topics for discussion.
Deadline for applications and papers: December 1, 1961.

All correspondence relating to the Congress should be addressed to the General Secretary of the National Organizing Committee of the U.S.S.R.: Prof. L. Shabad, Academy of Medical Sciences of the U.S.S.R., 14 Solyanka, Moscow.

U.S. GOVERNMENT PRINTING OFFICE: 1960